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(54) Title: CELLULAR GENES ENCODING RETINOBLASTOMA-ASSOCIATED PROTEINS

(57) Abstract

This invention provides an isolated nucleic acid molecule encoding a retinoblastoma-associated protein, and isolated proteins having transcriptional factor E2F biological activity and RB-binding activity. This invention also provides vectors comprising an isolated nucleic acid molecule encoding a retinoblastoma-associated protein, mammalian cells comprising such vectors, antibodies directed to the retinoblastoma-associated protein and hybridoma lines producing monoclonal antibodies to such protein. This invention further provides methods for using such antibodies diagnostically and prognostically.

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CELLULAR GENES ENCODING RETINOBLASTOMA-ASSOCIATED PROTEINS

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FIELD OF THE INVENTION

This invention relates to the molecular cloning of cellular genes encoding retinoblastoma-associated proteins. In a more specific aspect it relates to the identification of a gene with properties of the transcription factor E2F.

Throughout this application various publications are referenced by partial citations within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

The retinoblastoma gene (RB), the first tumor suppressor gene identified, encodes a nuclear phosphoprotein which is ubiquitously expressed in vertebrates (Friend, et al., Nature (London) 323:643-646 (1986); Lee, et al., Nature 329:642-645 (1987b); Fung, et al., Science 236:1657-1661 (1987)). Mutations of this gene which lead to inactivation of its normal function have been found not only in 100% of retinoblastomas but also in many other adult cancers including small cell lung-carcinoma (Harbour, et al., Science 241:353-357 (1988); Yokota, et al., Oncogene 3:471-475 (1988)), osteosarcoma (Toguchida, et al., Cancer Res. 48:3939-3943 (1988)), bladder carcinoma (Horowitz, et al., Science 243:937-940 (1989)), prostate

carcinoma (Bookstein, et al., PNAS USA 87:7762-7766 (1990a)) and breast cancer (Lee et al., Science 241:218-221 (1988)). Reconstitution of a variety of RB-deficient tumor cells with wild-type RB leads to suppression of their neoplastic phenotypes including their ability to form tumors in nude mice (Huang, et al., Science 242:1563-1566 (1988); Sumegi, et al., Cell Growth Diff. 1:247-250 (1990); Bookstein, et al., Science 247:712-715 (1990b); Goodrich, et al., Can. Res. 52:1968-1973 (1992); Takahashi, et al., PNAS USA 88:5257-5261 (1991); Chen, et al., Cell Growth Diff. 3:119-125 (1992)). These results provide direct evidence that RB protein is an authentic tumor suppressor.

RB performs its function at the early G1/G0 phase of the cell cycle as substantiated by several observations: first, the phosphorylation of RB, presumably by members of the Cdk kinase family (Lin, et al., EMBO J. 10:857-864 (1991); Lee, et al., Cell Cycle. 61:211-217 (1991)), fluctuates with the cell cycle (Chen, et al., Cell 58:1193-1198 (1989); Buchkovich, et al., Cell 58:1097-1105 (1989); DeCaprio, et al., Cell 58:1085-1095 (1989)); second, the unphosphorylated form of RB is present predominantly in the G0/G1 stage (Chen, et al., 1989, supra.; DeCaprio. et al., 1989, supra.)); third, microinjection of the unphosphorylated RB into cells at early G1 phase inhibits their progression into S phase (Goodrich, et al., Cell 67:293-302 (1991)). These observations suggest that RB may serve as a critical regulator of entry into cell cycle and its inactivation in normal cells could lead to deregulated growth.

How RB functions is the subject of intense inquiry. Two known biochemical properties of the RB protein have been described; one is its intrinsic DNA binding activity which was mapped to its C-terminal 300 amino acid residues (Lee et al., 1987b, supra.; Wang, et al., Cell Growth Diff. 1:429-437 (1990b)); another is its

ability to interact with several oncoproteins of the DNA tumor viruses (DeCaprio, et al., Cell 54:275-283 (1988); Whyte, et al., Nature 334:124-129 (1988); Dyson, et al., Science 243:934-937 (1989)). This interaction was mapped to two discontinuous regions at amino acids 379-545 and 575-678, designated as the T-binding domains (Hu, et al., EMBO J. 9:1147-1155 (1990); Huang, et al., EMBO J. 9:1815-1822 (1990)). Interestingly, mutations of the RB proteins in tumors were frequently located in these same regions (Bookstein and Lee, CRC Crit. Rev. Oncogenesis 2:211-227 (1991)). These results imply that the T-binding domains of RB proteins are functionally important and the interaction of RB with these oncoproteins may have profound biological significance. The identification of cellular proteins that mimic the binding of T to RB revealed a potentially complicated network. Several proteins including c-myc (Rustgi, et al., Nature 352:541-544 (1991)), Rb-p1, p2 (Defeo-Jones, et al., Nature 352:251-254 (1991)) and 8-10 other proteins (Kaelin, et al., Cell 64:521-532 (1991); Lee, et al., 1991, supra.; Huang, et al., Nature 350:160-162 (1991)) have been shown to bind to RB in vitro.

As the foregoing demonstrates, there clearly exists a pressing need to identify and characterize the cellular affiliates of the retinoblastoma gene. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding a retinoblastoma-associated protein, and isolated proteins having transcriptional factor E2F biological activity and RB-binding activity.

This invention further provides vectors such a plasmids and viruses comprising a DNA molecule encoding a

retinoblastoma-associated protein adapted for expression in a bacterial cell, a yeast cell, or a mammalian cell.

This invention provides a mammalian cell comprising a DNA molecule encoding a retinoblastoma-associated protein.

This invention provides an antibody capable of specifically binding to a retinoblastoma-associated protein. This invention also provides hybridoma cell lines that produce monoclonal antibodies and methods of using these antibodies diagnostically and prognostically.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of RB-sandwich screening. λgt11 cDNA expression libraries were plated and screened using the RB-sandwich containing purified p56-RB, anti-RB antibody, and alkaline-phosphatase conjugated secondary antibody. A and B, a diagram of the RB-sandwich screening. C and D, hybridized filters with the RB-sandwich (left halves of the filters) in which the positive signal indicates a RbAp-RB complex (C) or T-antigen-RB complex (D). The right halves of the filters were probed with the RB-minus sandwich.

Figure 2 shows binding of RbAps to RB <u>in vitro</u>. The cDNA insert from each clone (Ap4, 6, 9, 10, 11, 12, 15) was subcloned into the pFLAG plasmid and the lysates of FLAG-Ap fusion proteins were mixed with the GST-RB beads (R) or GST beads alone (C). The bound proteins were then analyzed by immunoblot using a monoclonal anti-FLAG antibody. The arrows indicate the FLAG-Aps bound to the GST-RB beads, which were detect d by the anti-FLAG antibody. BAP = FLAG bacterial alkaline phosphatase fusion protein.

Figure 3 shows cell cycle dependent expression of Ap12. Total RNA from CV1 cells synchronized at various stag s of the cell cycle was denatured and analyzed by formaldehyde gel electrophoresis. The RNA blot was hybridized with a ^{32}P-labeled Ap12 cDNA insert (G12). Lane 1, early G1; lane 2, G1/S boundary; lane 3, S phase (4 hours after aphidicolin release); lane 4, S phase (18 hours after replating starved cells); lane 5, M phase. The size of the mRNA (designated by an arrow) was determined by migration of the rRNA 28S and 18S, which were run on a parallel lane next to the RNA samples.

Figure 4 shows the restriction map and nucleic and amino acid sequences of Ap12. Clone A6, 2,492 nucleotides, was completely sequenced (SEQ ID NOS: 13-14). A: restriction map of Ap12 (A6) which has the longest open reading frame. G12 is the original Ap12 clone obtained by the RB-sandwich screening. A6 and B6 were isolated by rescreening of cDNA libraries. Only restriction sites used in the construction of Ap12 derivatives are shown. B: sequence of Ap12 and predicted amino acid sequence. The squares indicate the leucine repeats. Two putative Cdk phosphorylation sites are underlined.

Figure 5 shows that Ap12 binds specifically to the hypophosphorylated form of RB at regions similar to T. A, Lane 1: a Molt4 lysate immunoprecipitated using a monoclonal anti-RB antibody, mAb11D7. Lane 2: molecular marker. Lanes 3-5: Molt4 cell lysates ($5x10^6$ cells) were mixed with GST beads (lane 3), GST-Ap12 (lane 4) and GST-T (lane 5) beads. After washing, the RB bound to the GST fusions was analyzed by immunoblotting using a monoclonal anti-RB antibody, mAb11D7. B: a panel of RB mutant proteins expressed in a bacterial pET-T7 expression system. The T-binding domains are highlighted. C-D: the bacterially expressed wild type (pETRbc) or mutant RB proteins (pETB2, Ssp, Xs, M8, M6, M9, Nm) were mixed with

the GST-Ap12 (C) or GST-T (D) beads and the bound proteins were measured by Western blot analysis using a monoclonal anti-RB antibody, mAb245.

Figure 6 shows that the C-terminal region of Ap12 is required for RB-binding. A series of GST-Ap12 derivatives, P3, SH5, XH9, SX4, and XX4 were constructed (shown in panel B) and used for RB binding. The bacterially expressed pETRbc (wild type RB) was mixed with the GST-Ap12 beads and analyzed by Western blot analysis using a monoclonal anti-RB antibody, mAb245. The polypeptide encoding region for P3 is amino acids 362-476; SH5, aa 162-476; XH9, aa 1-476; SX4, aa 162-455; XX4, aa 1-455. The arrow indicates the position of p110-RB.

Figure 7 shows that Ap12 binds specifically to the E2F recognition sequence. The lysates prepared from the bacterially expressed derivatives of GST-Ap12 (P3, SH5, XH9) and GST-Ap9, GST-Ap15 and GST alone were used for DNA mobility shift assays. The probe was a DNA fragment containing two E2F recognition sites, which was ^{32}P-end-labeled by Klenow fill-in reaction. A: GST-Ap12SH5 binds to the E2F-specific sequence. As a positive control, a partially purified E2F protein from HeLa cells was also used. DNA fragments containing either the wild type E2F sites or mutated E2F sites were used as competitors. Lane 1: probe alone; Lane 2: E2F + probe; Lane 3: E2F + probe + wt competitor; Lane 4: E2F + probe + mutant competitor; Lane 5: SH5 + probe; Lane 6: SH5 + probe + wt competitor; Lane 7: SH5 + probe + mutant competitor. B: RB interacts with the Ap12-E2F DNA complex. Lane 1: probe alone; Lane 2: SH5 + probe; Lane 3: SH5 + p56-RB (0.25 μg), incubate for 15 minutes, followed by probe addition; Lane 4: p56-RB + probe; Lane 5: SH5 + probe; Lane 6: SH5 + probe for 15 minutes, then p56-RB was added. C: DNA binding domain of Ap12 is located at a region containing a potential bZIP motif. Lane 1: P3, 200ng; Lane 2: P3, 400ng; Lane 3,

SH5, 20ng; Lane 4: SH5, 40ng; Lane 5: XH9, 20ng; Lane 6: XH9, 40ng; Lane 7: GST alone, 200ng; Lane 8: GST-Ap9, 200 ng; Lane 9: GST-Ap15, 200ng.

Figure 8 shows that the C-terminus of Ap12 serves as an activation domain when fused to the GAL4 DNA binding domain in yeast. Fusion proteins of GAL4 (amino acids 1-147) and either G12 (AP12, amino acids 362-476), 12B6 (AP12, amino acids 22-476) or Rb2 (RB, amino acids 301-928) were expressed in yeast as detailed below. Plasmids were used to transform Y153 to tryptophan prototropy, and single colonies of each transformation were streaked on dropout media lacking tryptophan. Following 1 day of growth at 30°C, cells were analyzed for ß-galactosidase activity using a colony lift assay.

Figure 9 shows that Ap12 transactivates a promoter with E2F recognition sites. A: a diagram of the Ap12 cDNA expression vectors. PA, poly(A). B: transcriptional activation of a promoter with E2F recognition sequences. 10 μg of either $pA_{10}CAT$ or $pE2FA_{10}CAT$ was cotransfected with 10 μg of CMV-Ap12-Stu or CMV-Ap12-RH into monkey kidney CV1 cells. The cells were harvested after 48 hours and CAT activities were measured. CMV-E4 was cotransfected with the reporter plasmids as well as the reporter plasmids alone to serve as a control.

Figure 10 shows the partial nucleic acid sequence of clone Ap2. p = 5′ sequence (SEQ ID NO: 5); r = 3′ sequence (SEQ ID NO: 6).

Figure 11 shows the partial nucleic acid sequence of clone Ap8. p = 5′ sequence (SEQ ID NO: 7); r = 3′ s quence (SEQ ID NO: 8).

Figure 12 shows the partial nucleic acid sequence of clone Ap15. p = 5′ sequence (SEQ ID NO: 9); r = 3′ sequence (SEQ ID NO: 10).

Figure 13 shows the full length nucleic acid sequence of clone Ap4 (SEQ ID NO: 11).

Figure 14 shows the full l ngth nucleic acid sequence of clone Ap10 (SEQ ID NO: 12).

DETAILED DESCRIPTION OF THE INVENTION

The retinoblastoma protein interacts with a number of cellular proteins to form complexes which can be crucial for its normal physiological function. To identify these proteins, nine distinct gene cDNAs were cloned by direct screening of cDNA expression libraries using purified RB protein as a probe. Preliminary characterization of these clones indicates that a majority of these genes encode novel proteins. One of them, Ap12, expresses a 2.8 Kb mRNA in a cell cycle-dependent manner.

The longest cDNA isolate of Ap12 encodes a putative protein of 476 amino acids with several features characteristic of transcription factors. The C-terminal 114 amino acids of Ap12 binds to unphosphorylated RB in regions similar to where T-antigen binds and has transactivation activity. A region near the N-terminus contains a putative leucine zipper flanked by basic residues and is capable of specifically binding to an E2F cognate sequence. Expression of Ap12 in monkey kidney CV1 cells significantly enhanced E2F-dependent transcriptional activity. Although the E2F gene has not been cloned and its identity is based solely on the ability to recognize and bind to a specific DNA sequence, these results establish that the novel clones encode proteins with known properties of the transcription factor E2F and which bind RB.

Accordingly, the pres nt invention provides an isolated nucleic acid molecule encoding a retinoblastoma-

associated protein. As used herein, the term "isolated nucleic acid molecule" r f rs to a nucleic acid molecule that is in a form that do s not occur in nature. One means of isolating a human retinoblastoma nucleic acid molecule is to probe a human cDNA expression library with a natural or artificially designed antibody to retinoblastoma, using methods well known in the art (see Sambrook et al. Molecular Cloning: A Laboratory Manual 2d ed. (Cold Spring Harbor Laboratory 1989)) which is incorporated herein by reference). DNA and cDNA molecules which encode human retinoblastoma-associated polypeptides can be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources. The isolated nucleic acids can also be used to screen cDNA libraries to isolate other genes encoding RB-associated proteins.

The present invention provides soluble retinoblastoma-associated polypeptides that have DNA binding and RB binding activity. For the purposes of illustration only, nucleic acid sequences encoding the polypeptides are identified in Figures 4 and 10-14. The nucleic acid sequences encoding the soluble retinoblastoma-associated polypeptide are included within the sequences set forth in Figures 4 and 10-14.

As used herein "retinoblastoma-associated polypeptide" means a polypeptide having that has DNA binding as well as an RB-binding activity. Examples of retinoblastoma-associated polypeptides substantially the same as the amino acid sequence of clone Ap12, shown in Figure 4, or the amino acid sequence encoded by the nucleic acid sequences of clones Ap 2, 4, 8, 10 and 15, or active fragments thereof. As used herein, "an active fragment or biologically-active fragment" refers to any portion of the retinoblastoma-associated polypeptide shown in Figure 4, or that encoded by clones Ap 2, 4, 8, 10 and 15 shown in Figures 10-14. Methods of determining whether a

polypeptide can bind RB are well known to those of skill in the art, for example, as set forth herein.

As used herein, the term "purified" means that the molecule or compound is substantially free of contaminants normally associated with a native or natural environment. The purified polypeptides disclosed herein include soluble polypeptides. For example, the purified soluble polypeptide can be obtained from a number of methods. The methods available for the purification of proteins include precipitation, gel filtration, ion-exchange, reversed-phase, and affinity chromatography. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press 1990), which is incorporated herein by reference. Alternatively, a purified polypeptide of the present invention can also be obtained by well-known recombinant methods as described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual 2d ed. (Cold Spring Harbor Laboratory 1989), also incorporated herein by reference. An example of this means for preparing soluble retinoblastoma-associated polypeptide is to express nucleic acid encoding the retinoblastoma-associated polypeptide in a suitable host cell, such as a bacterial, yeast or mammalian cell, using methods well known in the art, and recovering the expressed soluble protein, again using methods well known in the art. The soluble polypeptide and biologically active fragments thereof can also be produced by chemical synthesis. Synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic polypeptide synthesizer and chemistry provided by the manufacturer. The soluble polypeptide can also be isolated directly from cells which have been transformed with the expression v ctors described below in more detail.

The invention also encompasses nucleic acid molecules which differ from that of the nucleic acid molecules shown in Figures, but which produce the same phenotypic effect. These alter d, but phenotypically equivalent nucleic acid molecules are referred to "equivalent nucleic acids." This invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule described hereinabove. This invention further encompasses nucleic acid molecules which hybridize to the nucleic acid molecule of the subject invention. As used herein, the term "nucleic acid" encompasses RNA as well as single- and double-stranded DNA and cDNA. In addition, as used herein, the term "polypeptide" encompasses any naturally occurring allelic variant thereof as well as man-made recombinant forms.

The invention further provides the isolated nucleic acid molecule operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off the nucleic acid molecule. Examples of such promoters are SP6, T4 and T7. Vectors which contain both a promoter and a cloning site into which an inserted piece of DNA is operatively linked to that promoter are well known in the art. Preferable, these vectors are capable of transcribing RNA _in vitro_ or _in vivo_. Examples of such vectors are the pGEM series (Promega Biotech; Madison, WI).

This invention provides a vector comprising this isolated nucleic acid molecule encoding a retinoblastoma-associated polypeptide. Examples of vectors are viruses, such as bacteriophages, baculoviruses and retroviruses, cosmids, plasmids and other recombination vectors. Nucleic

acid molecules are inserted into vector genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the insert DNA that correspond to a restriction site in the vector DNA, which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV) for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for in vitro transcription of sense and anti-sense RNA. Other means are available and one well known for those of skill in the art.

Also provided are vectors comprising a DNA molecule encoding a human retinoblastoma-associated polypeptide, adapted for expression in a bacterial cell, a yeast cell, a mammalian cell and other animal cells. The vectors additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, mammalian or animal cells so located relative to the DNA encoding retinoblastoma-associated polypeptide as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression

vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al., supra.). Similarly, a eucaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the polypeptide.

This invention provides a host cell, e.g. a mammalian cell, containing a nucleic acid molecule encoding a human retinoblastoma-associated polypeptide. An example is a mammalian cell comprising a plasmid adapted for expression in a mammalian cell. The plasmid has a nucleic acid molecule encoding a retinoblastoma-associated polypeptide and the regulatory elements necessary for expression of the polypeptide. Various mammalian cells may be utilized as hosts, including, for example, mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk- cells, etc. Expression plasmids such as those described supra can be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, DEAE-dextran, electroporation or microinjection.

Also provided are antibodies having specific reactivity with the retinoblastoma-associated polypeptides of the subject invention, such as anti-Ap12 antibody, or any antibody having specific reactivity to a retinoblastoma-associated polypeptide. Immunologically active fragments of antibodies are encompassed within the definition of "antibody." Identification of immunologically active fragments can be performed, for example, as detailed below. The antibodies of the

invention can be produced by any method known in the art. For example, polyclonal and monoclonal antibodies can be produc d by methods well known in the art, as described, for xample, in Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. The polypeptide, particularly retinoblastoma-associated polypeptide of the present invention, can be used as the immunogen in generating such antibodies. Altered antibodies, such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known to those skilled in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in Sambrook et al., *supra*, incorporated herein by reference. The antibodies can be used for determining the presence or purification of the retinoblastoma-associated polypeptide of the present invention. With respect to the detecting of such polypeptides, the antibodies can be used for *in vitro* diagnostic or *in vivo* imaging methods for diagnosing or prognosing pathologies associated with loss of functional RB protein.

Any of the above-identified novel compositions of matter may be combined with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" mean any of the standard carriers, such as saline, emulsion and various wetting agents. These compositions can be used for the preparation of medicaments for the treatment of pathologies associated with the loss of functional RB protein.

Immunological procedures useful for *in vitro* detection of the target retinoblastoma-associated polypeptide in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay,

agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in th art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Identification of RB-associated proteins. The simplest model for RB function is that relatively few target molecules which play central roles in cellular function are regulated by the retinoblastoma protein. Inactivation of RB by any one of three means, phosphorylation (Chen, et al., 1989, supra.; DeCaprio, et al., 1989, supra.), mutations (Shew, et al., PNAS USA 87:6-10 (1990)) or oncoprotein perturbation (DeCaprio, et al., 1988, supra.; Goodrich, et al., 1991, supra.; Whyte, et al., 1988, supra.), could potentially uncouple RB connections and lead to deregulated growth. Until this report, there were, indeed, only a limited number of molecules that were known to be capable of interacting with RB, such as two proteins of unknown function, p1 and p2, the myc protein and 8-10 other unidentified proteins. To genetically and biochemically dissect the RB network, it is essential to identify as many of the genes encoding interactive partners of RB as possible. To maximize the cloning probability, two different approaches were undertaken. One approach was to use a two-hybrid method developed by Field and his colleagues (Fields and Sung, Nature 340:245-246 (1989)) based on the yeast GAL4 system to select for protein-protein interaction *in vivo*. The other approach, described herein, was to use an RB-sandwich to scr en λgt11 cDNA expression libraries. The advantage of using this one-step RB-sandwich procedure is its simplicity, directness, and the clone isolated should encode a fusion protein that would directly interact with

RB in the absence of potential bridging proteins. Screening was performed using SV40 large T antigen as a positive control. A λgt11 phage expressing T antigen was constructed for this purpose and the association between RB and T can be readily detected by this method.

Using this approach, 9 clones were isolated. All the proteins encoded by these clones are located in the nucleus. This is an important criteria for any protein that could interact with RB in a biologically significant manner, since the interaction probably would occur in the nucleus (Lee, et al., 1987b, supra.).

Transcription factors as targets of regulation by the RB protein. If the cellular function of RB is to restrict entry of cells into G1 (Goodrich, et al., 1991, supra.), the genes important for G1 progression and entrance into S phase should be regulated directly or indirectly by RB. The transcription factor E2F is known to associate with RB in a cell-cycle-dependent manner (Mudryj, Cell 28:1243-1253 (1991); Shirodkar, Cell 68:157-166 (1992)), with a tight association being prevalent in the G0/G1 stage but not in S or M phases. There are several genes including myc, DHFR, and myb that may be subject to E2F transcriptional control (Hiebert, et al., PNAS USA 86:3594-3598 (1989); Mudryj, et al., EMBO J. 9:2179-2184 (1990)). It is reasonable to propose that RB sequesters E2F in the G0/G1 stage in an inactive conformation. Its release from the RB complex allows it to assume an active conformation that is capable of influencing its target genes through interactions with E2F DNA-binding sites and the general transcriptional machinery. An important challenge is to determine the identity of the E2F target genes and to ascertain their role in the control of the cell cycle.

There is increasing evidence to support this simple model of RB function, which is now further supported by the finding that, in the collection of 9 newly cloned RB-associated proteins, one is a known eukaryotic upstream binding factor (UBF) which recognizes and binds to the ribosomal RNA promoter, and activates transcription mediated by RNA polymerase I through cooperative interactions with SL1 (Jantzen, et al., Nature 344:830-836 (1990)), and another, Ap12, has properties consistent with those proposed for the E2F transcription factor. The accumulation of Ap12 mRNA around six hours post stimulation with serum coincides with the pattern of expression of delayed-early growth response genes (Lau and Nathans, "Genes induced by serum growth factors" In The Hormonal control regulation of gene transcription, ed. P. Cohen & J.G. Foulkes, Elsevier Science Publishers, pp. 257-293 (1991)). The maximal level of Ap12 mRNA accumulates at the G1/S boundary, establishing that it has a role in controlling cells of entry into S phase. Also, the protein binds only to unphosphorylated RB at domains similar to those bound by T. Most interestingly, Ap12 recognizes the E2F cognate sequence and transactivates the promoter carrying such specific sequence.

Ap12 encodes a putative bZIP transcription factor. From the preliminary characterization of this gene, the putative protein deduced from the longest open reading frame is 476 amino acids in length although the initiating methionine has yet to be defined. The predicted molecular weight of the putative protein is about 51 kd which is close to the 60 kd protein immunoprecipitated by the anti-Ap12 antibody. The C-terminal region of Ap12 which binds to RB protein and has a transactivation activity, is very acidic, a hallmark of the transactivation domain of several known transcription factors such as GAL4 and VP16 (Sadowski, et al., Nature 335:563-564 (1988); Mitchell and Tjian, Science 245:371-378 (1989)). The DNA

binding domain appears to be located at the middle region of the protein which features a putative leucine zipper motif flanked by stretches of basic amino acids. Since Ap12 has most of the features that are characteristic of E2F, it can be considered to either encode E2F or a protein in the E2F family. Thus it is likely that E2F is also a bZIP protein which is intriguing since this is a class of transcription factors intimately involved in cell growth (e.g., fos and jun) and differentiation (e.g., C/EBP). Another hallmark of the bZIP family is a propensity to form a diverse array of heterodimeric associations among its members which adds a new layer of regulation to the control of E2F.

This vast array of possibilities presents an almost unlimited opportunity for the cell to intricately regulate the proteins involved in fine control of the cell cycle. The availability of the Ap12/E2F clone will facilitate the further elucidation of the connection between RB, E2F and cellular proliferation.

To identify the cellular affiliates of RB and to initiate the elucidation of the RB interactive cellular network, several approaches were taken to clone genes encoding RB-associated proteins. Described herein are the results from one of these approaches: screening of λgt11 expression libraries using RB as a probe. Nine distinct genes were cloned, one of which, Ap12, has characteristics which suggest that it encodes the transcription factor E2F. Clones Ap 2, 4, 8, 10, 12 and 15 all encode RB-associated proteins and are all involved in cell cycle control.

Identification of RB-associated proteins (RbAps). Two λgt11 cDNA expression libraries were constructed and screened using the purified p56-RB protein (amino acids 376-928) which includes both T-binding domains and entire C-terminal region (Lee, et al., 1991, <u>supra.</u>) as probe.

This probe is referred to as a RB-sandwich since it contains RB protein, rabbit anti-RB antibody, (0.47) (Wang, et al., Cell Growth Diff. 1:233-239 (1990a)), and alkaline phosphatase conjugated goat anti-rabbit IgG. (see Materials and Methods). Figure 1 illustrates a diagram of the sandwich screening strategy (1A and 1B). Since the association of RB and SV40 T-antigen is well documented (DeCaprio, et al., 1988, supra.), a λgt11 phage expressing T-antigen was constructed and screened using the RB-sandwich to serve as a positive control (shown in Figure 1-D). As an example (Figure 1-C), one of the clones' (Ap12) fusion product, was readily detected by this method. One half of each filter was used for binding to the RB-sandwich and the other half to the sandwich minus RB protein. The latter probe served as a control for the background binding due to any cross-reaction of the RB antibody or goat anti-rabbit antibody with bacterial proteins. After 5 rounds of screening of 1 x 10^6 recombinant phage, 12 clones emerged as candidate genes encoding RB-associated proteins. These clones are designated RbAp1, 2, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15.

These 12 putative RbAp cDNAs were subcloned into the pGEM plasmid and a partial sequence of 500 to 600bp from each clone was obtained. A comparison with known gene sequences present in the GENBANK database, RbAp1, 2, 4, 8, 10, 12, 13, 14, 15 appear to be novel genes that contain no significant homology to any known genes. However, three clones matched previously identified genes: RbAp6 is identical to nuclear lamin C (McKeon et al., Nature 319:463-468 (1986); Fisher et al., PNAS USA 83:6450-6454 (1986)); RbAp9 encodes a product partially homologous to the ß subunits of G protein (Gullemont et al., PNAS USA 86:4594-4598 (1989)); and RbAp11 codes for the upstream binding factor (UBF) that binds to the ribosomal RNA gene promoter (Jantzen, et al., supra.). Cross-hybridization and sequencing data showed that RbAp1, 10, 13, and 14 are

identical. Table 1 summarizes the preliminary characterization of all the cloned RbAps.

RbAp clones 2, 4, 8, 10, 12, and 15 are targets for RB, $p110^{RB}$, binding and all function in cell cycle control. It is possible that the retinoblastoma-associated proteins encoded by the RbAp clones are positive elements for cell proliferation. Rb binds to the protein products of these clones and, therefore, inhibits their proliferative function. As a result, the RbAp protein products cannot function positively and, therefore, are unable to promote cell cycle progression. Alterations in the RbAp ability to bind RB can result in an oncogenic effect. Assays detecting such alterations and/or mutations could determine malignancy and function as diagnostic tools for hyperproliferative diseases. Examples of hyperproliferative pathologies include, but are not limited to thyroid hyperplasia, psoriasis, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, benign prostatic hypertrophy and various leukemias and lymphomas. The present invention also provides antagonists of such altered and/or mutated RbAps for use in therapeutics for cancer and other hyperproliferative pathologies.

Table 1. Initial characterization of RB-associated proteins. The size of cDNA of each clone was determined by the EtBr staining of the agarose gel after digestion of the phage DNA with EcoRI. The size of mRNAs was measured by the RNA blot analysis using 28s and 18s rRNA as markers. The partial sequence from each clone was used to search GENBANK database to determine the identity of the clones. The nuclear localization was determined by immunostaining and cell fractionation (data not shown). nd = not determined.

RbAp	Length of cDNA(kb)	Size of mRNA(kb)	*in vitro* Binding	Identity	Subcellular Localization
1,10,13,14	2.8	7.1	+	Novel	Nucleus
2	1.6	3.6	nd	Novel	nd
4	1.7	6.7	+	Novel	Nucleus
6	1.5	2.1	+	Lamin C	Nucleus
8	1.8	6.9	nd	Novel	nd
9	0.7	1.3	+	GB-like	Nucleus & Membrane
11	1.5	3.2	+	UBF	Nucleus
12	1.4	2.8	+	Novel	nd
15	1.5	6.5	+	Novel	Nucleus

Binding of RbAps to RB *in vitro*. To confirm the association of RB protein with RbAps, the cloned cDNA inserts were subcloned into the plasmid pFLAG (IBI). This plasmid is designed for expressing Flag-fusion proteins in bacteria which can then be detected using an antibody against the Flag segment of the fusion. To facilitate the binding assay, the p56-RB was fused with the glutathione S-transferase (Gst) gene, expressed and purified by glutathione agarose chromatography (Gst-RB) (Smith and Johnson, Gene 67:31-40 (1988)). To perform the RB-binding assay, the FLAG-Ap lysates were mixed with the Gst-RB or Gst beads alone (no RB). As an additional n gative

control, FLAG-BAP (bacterial alkaline phosphatase) was also mixed with the Gst and Gst-RB beads. After extensive washing, the bound fusion proteins were eluted and analyzed by Western blotting using the anti-FLAG monoclonal antibody. The results demonstrate that all RbAps examined are able to bind to the Gst-RB beads but not to the control Gst beads (Figure 2). Among these clones, the binding affinity varied from Ap15, the weakest, to Ap12, the strongest.

The level of Ap12 mRNA is regulated during the cell cycle. Since Ap12 consistently showed the strongest binding signal during screening, it was selected for further study. The clone has an insert of 1.4 kb with a about 1.0 kb untranslated region and an open reading frame of 114 amino acids. RNA blot analysis was performed to determine the size of the mRNA and its pattern of expression during cell cycle progression. Normal monkey kidney CV1 cells were plated in fresh medium with 10% serum in the presence of Lovastatin for 36 hours (to arrest the cell in G1 phase) (Jakobisiak, et al., PNAS USA 88:3628-3632 (1991); Keyomarsi, et al., Can. Res. 51:3602-3609 (1991)) or aphidicolin (10 μg/ml) for 16 hours (to arrest the cells at the G1/S boundary), then released for 4 hours (to synchronize the cells in S phase) or incubated in the presence of nacodazole for another 16 hours (to allow the cells to progress to M phase) (Goodrich, et al., 1991, supra.). Total RNA from each stage was prepared for blot analysis using the Ap12 cDNA as a probe. A 2.8 kb mRNA was detected at the G1/S boundary and in S phase, but was undetectable in early G1 or M phase (Figure 3). As a control, the expression pattern of Ap9 does not change during the cell cycle. Consistent with this observation, an increase of Ap12 mRNA expression was observed between 2 and 6 hours after serum stimulation. These findings establish that Ap12 can be involved in cell cycle progression.

Sequence analysis of Ap12. It is apparent that the initial Ap12 cDNA clone (G12) was shorter than the size of its corresponding mRNA. The cDNA libraries were rescreened and several longer clones were isolated, among them, two clones, A6 and B6, together with the original clone (G12) were further characterized (Figure 4). The longest open reading frame from the 2,492 nucleotides encodes a putative protein of 476 amino acids. Distinctive features of the putative protein include the C-terminus 100 amino acids that are very acidic, and an N-terminal 43 amino acid region dominated by 15 proline residues. Following the proline-rich region are typical leucine repeats (Landschulz, et al., Science 240:1759-1764 (1988); Vinson, et al., Science 246:911-916 (1989)), flanked by stretches of basic amino acids, suggesting a potential DNA-binding domain. These features are indicative of several different classes of eukaryotic transcription factors. In addition, a stretch of amino acids (LXSXE----- DDE) (SEQ ID NO: 1) at position 389-411 resembles the sequences of T-antigen which are responsible for binding to RB protein (DeCaprio, et al., 1988, supra.). Furthermore, there are two potential phosphorylation sites for Cdk kinase (Shenoy, et al., Cell 57:763-774 (1989)) at amino acids 159-161 (KSP) and 346-349 (SPGK) (SEQ ID NO: 2), which could modulate the function of this protein.

Ap12 binds only the hypophosphorylated form of RB at regions similar to those required for binding of SV40 T-antigen. To analyze the RB-binding properties of Ap12, the original clone (G12) was expressed as a Gst-fusion protein (P3) and purified by glutathione agarose chromatography. This fusion protein was used to test the binding of the Ap12 protein to full-length RB prepared from a cellular lysate of Molt4 cells, that expresses both hyper- and hypo-phosphorylated forms of the RB protein. Two additional controls were included in this experiment: one was a Gst-T-antigen fusion protein as a positive control and the other

was Gst alone as negative control. As shown in Figure 5A, the P3 protein binds only to the hypophosphorylated form and the binding affinity is very similar to that of T. Gst alone binds no detectable RB protein. To define which domain of RB is binding to Ap12, a panel of RB mutants expressed in the bacterial pET-T7 expression system (Studier et al., Meth. Enzymol. 185:60-89 (1990)) were mixed with the P3 beads or in parallel, with Gst-T beads. The amount of wild type or mutated RB proteins bound to the beads was determined by Western blot analysis using a monoclonal anti-RB antibody (mAb245). As shown in Fig 5C and 5D, the mutated RB defective in binding to T also failed to bind to Ap12. These results indicate that both Ap12 and T bind to the unphosphorylated form of RB in similar regions, showing that the Ap12-RB association is biologically significant.

The C-terminal region of Ap12 is required for binding to RB. Since the initial P3 fusion protein which contains 114 amino acids of Ap12 binds to RB, additional experiments were designed to map the region of Ap12 required for binding to RB. Four Gst-Ap12 fusion proteins with different N-terminal or C-terminal deletions were constructed, XH9 contains the entire coding sequence of the Ap12 cDNA and SH5 (from Sma I to Hind III) contains the C-terminal 314 amino acids. XX4 and SX4 are derived from XH9 and SH5, respectively, and contain a deletion of 21 amino acids at the C-terminus. The bacterially expressed RB protein (pETRbc) was mixed with these Gst-Ap12 derivatives and analyzed by Western blotting, as described above. Xh9, SH5 and P3 bind to RB with similar affinity, suggesting that the N-terminal sequence of Ap12 contributes little to RB-binding. However, XX4 and SX4, that both have 21 amino acids deleted from the C-terminus but contain the (LXSXE---DDE) sequence (DeCaprio, et al., 1988, supra.; Phelps, et al., J. Virol. 66:2418-2427 (1992)), failed to bind RB (Figure 6). Together, these results indicate that the C-

terminal region of Ap12 is required for binding to RB and the (LXSXE---DDE) sequence alone is not sufficient for binding, suggesting that the mode of RB-Ap12 interaction may be different from that of RB-T or RB-E1A interaction.

Ap12 binds specifically to the E2F recognition sequence. Since it has been shown that RB forms a complex with the transcription factor E2F (Bagchi, et al., Cell 65:1063-1072 (1991); Bandara, et al., Nature 352:249-251 (1991); Chellappan, et al., Cell 65:1053-1061 (1991)), and Ap12 has a potential DNA-binding domain, experiments were performed to determine whether Ap12 could interact with an E2F binding site. The bacterially expressed Gst-Ap12 (SH5) fusion protein was used in the DNA mobility shift assay of a DNA fragment containing two E2F recognition sites using previously described conditions (Yee, et al., Mol. Cell Biol. 9:578-585 (1989)). As shown in Figure 7A, SH5 binds that probe specifically since the complex is effectively competed with the unlabeled DNA fragment containing the wild-type E2F cognate sequence but not by a mutated sequence that differs from the wild type by only two nucleotides (Yee, et al., supra.). As a positive control, partially purified E2F protein from HeLa cells specifically binds to the DNA probe as expected.

To determine if RB is able to interact with the Ap12-DNA sequence specific complex, purified p 56-RB protein was included in the DNA mobility shift assay. The experiments were performed in two ways, either SH5 was mixed with RB then added to the E2F probe (Fig 7B, lane 3) or the fusion protein was bound to the E2F probe first followed by addition of RB (Figure 7B, lane 6). In either case, the Ap12-DNA complex was super-shifted to more slowly migrating positions by adding RB, indicating that RB has the ability to interact with the specific Ap12-DNA complex. These results show that the Ap12 protein has a DNA-binding

as well as a RB-binding activity similar to that shown for E2F.

To determine whether the region containing the leucine repeats is required for DNA binding, three Gst-Ap12 fusion proteins, P3, SH5 and XH9 were chosen for DNA mobility shift assays. As shown in Figure 7C, SH5 and XH9 which contain the putative leucine zipper and stretches of basic amino acid residues (bZIP) (Vinson et al., supra.) bound to the E2F recognition sequence whereas the C-terminal region of Ap12 (P3) did not. In addition, some other controls, Ap9, Ap15 and Gst alone, also tested negative. This result demonstrates that a region containing the putative bZIP motif is necessary for the Ap12-DNA specific interaction.

The C-terminus of AP12 can function as a transactivation domain. Highly acidic, amphipathic alpha-helical regions commonly serve as a activation domains in eukaryotic transcription factors (for review see Mitchell and Tjian, supra.). The C-terminal region of AP12 also displayed these characteristics, suggesting that it may function in an analogous manner. To test this, AP12 sequences encoding either amino acids 22-476 or the C-terminal 114 amino acids (362-476) were fused to those for the DNA binding domain of the yeast GAL4 protein (amino acids 1-147) (Keegan, et al., Science 231:699-704 (1986)) present on a yeast expression vector. While this GAL4 fragment can bind specifically to its recognition site (UAS_G) (Keegan, et al., supra.), it lacks an activation domain. Therefore, the chimeric protein relies on the fused segment to provide activation functions in order to direct transcription from a UAS_G containing promoter. Several such fusions involving mammalian activators have been shown to be functional in yeast, including p53 (Fields and Jang, Science 249:1046-1051 (1990)). As shown in Figure 8, following transformation of yeast strain

harboring the E. coli lacZ gene under UAS_G control, both GAL4-AP12 fusions were able to activate transcription of the reporter as evidenced by ß-galactosidase activity wher as the GAL4-RB control was not. This result indicates that AP12 does contain an activation domain, and that the C-terminal 114 amino acids are sufficient for this function.

Expression of Ap12 in CV1 cells transactivates a promoter with E2F recognition sequences. To determine whether Ap12 can activate transcription in an E2F binding site-dependent manner, two plasmids, CMV-Ap12-Stu and CMV-Ap12-RH, were constructed to express the Ap12 in mammalian cells under the control of a cytomegalovirus(CMV)-IE promoter (Neill, et al., <u>J. Virol.</u> 65:5364-5373 (1991)) (Figure 9A). Two reporter plasmids, $pE2FA_{10}CAT$ with two E2F sites upstream of the CAT reporter gene, and $pA_{10}CAT$ containing no E2F binding sites (Yee, et al., <u>supra.</u>), were used for this assay. Figure 9B showed that the expression of either CMV-Ap12-Stu or CMV-Ap12-RH significantly enhanced CAT activity when $pE2FA_{10}CAT$, but not $pA_{10}CAT$, was cotransfected. Expression of CMV-E4 has no apparent effect when compared with the control cells which were only transfected with the reporter plasmid. These data suggested that Ap12 encodes a functional transcription factor which activates promoters with E2F recognition sequences.

Isolation of cellular genes encoding Rb-associated proteins. Two cDNA libraries were constructed from poly A^+ RNA isolated from HeLa cells and Saos2 cells by previously described methods (Sambrook et al., <u>supra.</u>). The double stranded cDNAs were size fractionated by using Sepharose Cl-4B chromatography and were ligated to λgt11 arms. The size of the <u>in vitro</u> packaged libraries was 2.0 x 10^7 recombinants for HeLa cells and 1.5 x 10^7 for Saos2 cells with the average size of inserts being 1.6 kb. The

cDNA libraries were plated on one hundred 150mm dishes at 1-2 x 10^4 recombinants per dish and incubated at 42°C until plaques just became visible (3.5 hours), and then transferred to the nitrocellulose filters saturated with IPTG (10 mM) for overnight at 37°C. The filters were denatured and renatured in 6M guanidine HCl and incubated with the RB-sandwich probe in binding buffer (25 mM Hepes, pH 7.5, 50 mM NaCl, 5mM $MgCl_2$, 5 mM DTT, 0.1% NP-40, 5% milk, 1 mg/ml BSA) for 4 hours at 4°C. The RB-sandwich was prepared by mixing 1 μg of purified bacterially expressed p56-RB (Huang et al., 1991, supra.), 100 μl of preabsorbed polyclonal anti-RB antibody (anti-RB 0.47, 1:100 dilution) and 1 μl of alkaline-phosphatase conjugated secondary antibody (1:1000 dilution) per ml of binding buffer, incubated at 4°C for 2 hours. The RB-minus control sandwich was prepared by mixing the RB antibody and the secondary antibody and used as a control to eliminate the clones cross-reacted with the anti-RB antibody. The bound filters were then washed in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) 5 times, 3 minutes each and color developed in BCIP/NBP (Promega, WI). Positive clones from the initial screening were picked and subjected to second and third rounds of screening. The clones that consistently showed positive signals with the RB-sandwich but not with the RB-minus sandwich were then selected for fourth and fifth rounds of screening by plating at low density mixed with control phages to ensure homogenous isolates obtained which gave strong positive signals over the background.

Plasmid construction and fusion protein expression. The cDNA inserts of RbAps clones were subcloned into the pGEM1 for sequencing analysis. To express RbAp fusion proteins in vitro, the cDNA inserts were reconstructed in-frame into the pFLAG fusion protein expression syst m (IBI). The expression of the FLAG fusion proteins wer induced by 0.2 mM of IPTG and the bacterial

lysates were prepared by two rounds of freeze-and-thaw followed by sonication in lysis buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM DTT, 0.2% NP-40, 1 mM PMSF, 1 μg/ml Leupeptin, 5 μg/ml Aprotinin, 1 μg/ml Antipain) and were clarified by centrifugation. To express the RB protein in vitro, the p56 version of the RB cDNA fragment (aa 377-928) was subcloned into a plasmid expressing glutathione S-transferase (GST) fusion protein pGEX-2T (Smith and Johnson, supra.) and the bacterially expressed GST-RB fusion was prepared and purified using GST agarose beads.

In vitro binding assay. Bacterial lysates (100 μl) containing about 0.5 μg of the FLAG-RbAps were mixed with 20 μl of the GST-RB beads or GST beads carrying 1-2 μg of the fusion protein in 400 μl lysis buffer B at 4°C for 60 minutes. The bound beads were subsequently washed 5 times in 1 ml PBS/0.2%NP-40 and the protein complex was boiled in SDS loading buffer. The bound FLAG fusion proteins were then analyzed by SDS polyacrylamide gel electrophoresis, immunoblotted and probed with an anti-FLAG monoclonal antibody (IBI).

Construction of mutated RB proteins expressed in the bacterial pET-T7 system. In addition to pETRbc, pETM6 and pETM9 (Huang et al., 1991, supra.), pETB2, pETSsp and pETM8 were constructed by cloning AhaII-BamHI fragments from pB2, pSsp and pM8 (Huang et al., 1990, supra.) into the corresponding pET expression vector. The bacterial lysates were prepared as described in previous section.

Construction of GST-RbAp12 fusion proteins. The DNA fragments derived from RbAp12 clones were subcloned into the GST fusion plasmids. GST-P3 was constructed by cloning the Eco RI-Sph I fragment from the original C-terminal 1.3kb cDNA (G12) into pGEPK, a derivative from pGEX-2T (Smith and Johnson, supra.). GST-SH5 contains the

SmaI-HindIII fragment from clone B6 and GST-XH9 contains the EcoRI-HindIII fragment of clone A6 that contains the entire coding sequence. GST-SX4 and GST-XX4 are derived from GST-SH5 and GST-XH9, respectively, but the C-terminal XhoI-HindIII fragment is deleted.

RNA Blot Analysis. Total RNA extracted by the guanidine isothiocyanate-CsCl method (Sambrook et al., <u>supra.</u>) was denatured in 50% formamide, 2.2M formaldehyde, 20 mM Na borate (pH 8.3) and analyzed by 1.0% agarose gel electrophoresis. The RNA was then transferred to Hybond paper (Amersham) and the blot was immobilized by UV crosslinking. Prehybridization and hybridization were carried out in 50% formamide, 5x SSPE, 5x Denhardt's, 1% SDS and 100 μg/ml salmon sperm DNA and hybridization was performed in presence of ^{32}P-labeled 1.3 kb RbAp12 insert DNA at 45°C for 18 hours. The initial washing was carried out in 2x SSC, 0.1% SDS at room temperature and the final washing was in 0.1x SSC, 0.1% SDS at 65°C for 45 minutes.

DNA gel mobility shift assay. The insert from plasmid containing two E2F recognition sequences (TTTCGCGC---GCGCGAAA) (SEQ ID NO: 3) was used as a probe for the gel mobility shift assay and also served as a competitor. A plasmid containing a mutated E2F site (TTTAGCGC---GCGCTAAA) (SEQ ID NO: 4) (Huang et al., <u>DNA and Cell Biol.</u> 11:539-548 (1993)), which does not bind to E2F, was also used as a competitor. The assay was performed as described previously (Yee et al., <u>supra.</u>). The diluted GST-Ap12 bacterial lysates (20ng for SH5 and XH9 fusion proteins, 200ng for P3, Gst, GstAp9 and GstAp15) were incubated with 1x binding buffer (20 mM Hepes, pH 7.6, 1 mM $MgCl_2$, 0.1 mM EGTA, 40 mM KCl, 10% glycerol), 0.1% NP40, 1mg/ml salmon sperm DNA at room temperature for 15 minutes and the ^{32}P-end-labeled (Klenow fill-in) probe was added for another 30 minutes. The protein-DNA complexes were analyzed by 4% acrylamide gel electrophoresis in 0.25x TBE buffer at 4°C.

Yeast Expr ssion Vector and Strain. The xpression plasmid used in yeast was based on the pAS1 vector. Briefly, the plasmid contains the ADH1 promoter driving expression of the GAL4 DNA-binding domain followed by a downstream polylinker. The vector also carries the 2μ origin and TRP1 gene for maintenance and selection in yeast. pAS/G12 was constructed by subcloning the EcoRI fragment isolated from G12 into the unique EcoRI site in pAS1. Similarly, pAS/12B6 was built using the EcoRI fragment from p12B6 and subcloning into the pAS1 EcoRI site. pASRb2 will be described elsewhere. The Saccharomyces cerevisiae strain used was Y153 (MATa, trpl-901, leu2-3, -112, ade2-101, ura3-52::URA3 (GAL1-lacZ), MEL (GAL1-lacZ).

Yeast Transformation and ß-galactosidase Assay. Yeast transformation was carried out using the LiOAc method as described previously (Schiestl and Gietz, Curr. Genet. 16:339-346 (1989)). After transformation, cells were plated on synthetic dropout media lacking tryptophan to select for the presence of the plasmid. Following 2-3 days growth at 30°C, single colonies from each transformation were streaked onto another selective plate and allowed to grow an additional 24 hours. The colony color ß-galactosidase activity assay was then performed as described (Breeden and Nasmyth, Quant. Biol. 50:643-650 (1985)) except the nitrocellulose filters were submerged in liquid nitrogen for about 30s-60s to permeabilize the cells, then thawed at room temperature before overlaying on Whatman filters saturated with LacZ-X-Gal solution (Breeden and Nasmyth, supra.). The color developed in about 20 minutes in the case of the AP12 clones. No color change was observed with the pAS/Rb2 clone even after overnight exposure.

Transi nt Transfecti n Assay. The transfections were carried out with CV1 cells by conventional calcium

phosphate precipitation method. The plasmid pCMVAp12Stu was construct d by cloning the StuI fragment from clone A6 into the SmaI site of pCMV and plasmid pCMVAp12RH contains the EcoRI-HindIII fragment of clone B6. The plasmid pCMVE4 was used as a control. The CMV constructs were cotransfected with plasmids pE2FA$_{10}$CAT (containing two E2F binding sites) and pA$_{10}$CAT (containing no E2F binding sites) with the same number of cells (5x10^6) and the CAT activities were measured after 48 hours as described previously (Gorman et al., Mol. Cell Biol. 2:1044-1051 (1982)).

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims which follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM

(ii) TITLE OF INVENTION: CELLULAR GENES ENCODING RETINOBLASTOMA-ASSOCIATED PROTEINS

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER:
(B) FILING DATE: 19-NOV-1993
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(viii) ATTORNEY/AGENT INFORMATION:
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(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 619-535-8949

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Leu Xaa Ser Xaa Glu Asp Asp Glu
1               5
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Pro Gly Lys
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTCGCGCGC GCGAAA 16

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTTAGCGCGC GCTAAA 16

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 178 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCTTGACC TTGCTGGGAA TGCTCGGTCA GACAAGGGCA GCATGTCTGA AGACTGTGGG 60

CCAGGAACCT CCGGGGAGCT GGGCGGCTGA GGCGATCAAA ATTGAGCCAG AGGATCTGGA 120

CATCATTCAG GTCACCGTCC CAGACCCCTC GCCAACCTCT GAGGAAATGA CAGACTCG 178

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 151 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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TTTTTTACTT ATTTAAAAAG GCCTTGGTGG CAGGAATATA GTGTAAAAAT CATTGGAAAA     60
ACTAAAAGGC ATCGATACAT ATCCGAATAT ACATTTTGTA CATAAATTAC ATTTCCTTTA    120
GTCTTTCTGA GTGAGGTCCT GATTCAGTAC T                                   151
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 255 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
TTTACGACAG AGCACTATTG CCAAGCGTTC AAATGCAGCA CCATTAAGTA ACACAAAAAA     60
AGCATCTGGG AAGACTGTAT CTACTGCTAA AGCAGGAGTG AAACAACCAG AAAGGAGTCA    120
GGTTAAAGAA GAAGTATGTA TGTCACTGAA ACCTGAGTAC CATAAGGAGA ATAGAAGGTG    180
CAGCCGAAAT AGCGGACAAA TTGAAGTGGA TACCTGAAGT ATCAGTGTCT TCAAGTCATT    240
CTTCAGTGTC ATCTT                                                     255
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 245 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
GAATTCAACT GTAGCTTGGT TTTCCAAAGT ATCTGGATCT AGTATTTCAG TCTTTTTGTC     60
TTCTTCAGCA CAACATTTTA CACAGACATA TTCTTTGTCT TCCTCGCCCA TCTGCTGTGC    120
TTGAGAAAGA CTTAACCCAA CACAATCACC ATGAAACCAG TCATCACATC TCCACAGCCA    180
ACCATAACTG TTGCATGTGT TTTTGCAAAC CACACTGTTG CTGGAGTCAC ATATATTCGT    240
TCAAT                                                                245
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 688 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GAATTCAGTG GAGCACCAGT AGAAGGTGCA GGAGAAGAGG CATTGACTCC ATCAGTTCCT    60
ATAAATAAAG GTCCCAAACC TAAGAGGGAG AAGAAGGAGC CTGGTACCAG AGTGAGAAAA   120
ACACCTACAT CATCTGGTAA ACCTAGTGCA AAGAAAGTGA AGAAACGGAA TCCTTGGTCA   180
GATGATGAAT CCAAGTCAGA AAGTGATTTG GAAGAAACAG AACCTGTGGT TATTCCAAGA   240
GATTCTTTGC TTAGGAGAGC AGCAGCCGAA AGACCTAAAT ACACATTTAA TTTCTCAGAA   300
GAAGAGGATG ATGATGCTGA TGATGATGAT GATGACAATA ATGATTTAGA GGAATTGAAA   360
GTTAAAGCAT CTCCCATAAC AAATGATGGG GAAGATGAAT TTGTTCCTTC AGATGGGTTA   420
GATAAAGATG AATATACATT TTCACCAGGC AAATCAAAAG CCTCACCAGA AAAATCTTTG   480
CATGACAAAA AAAGTCAGGA TTTTGGAAAT CTCTTCTCAT TTCCTTCATA TTCTCAGAAG   540
TCAGAAGATG ATTCAGCTAA ATTTGACAGT AATGAAGAAG ATTCTGCTTC TGTTTTTTCA   600
CCATCATTTG GTCTGAAACA GACAGATAAA GTTCCAAGTA AAACGGTAGC TGCTAAAAAG   660
GGAAAACCGT CTTCAGATAC AGTCCCTA                                      688
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
GCAATGTTTA ATTAAGTGGG GAAAGAGCAC AAACATTTTT CAACAAATAC TTGTGTTGTC    60
CTTTTGTCTT CTCTGTCTCA GACCTTTTGT ACATCTGGCT TATTTTAATG TGATGATGTA   120
ATTGACCGTT TTTTATTATT GTGGTAGGCC TTTTAACATT TTGTTCTTAC ACATACAGTT   180
TTATGCTCTT TTTTACTCAT TGAAATGTCA CGTACTGTCT GATTGGCTTG TAGAATTGGT   240
TATAGACTGC CGTGCATTAG CACAGATTTT AATTGTCATG GTTACAAACT ACAGACCTGC   300
TTTTTGAAAT GAAATTTAAA CATTAAAAAT GGAACTGTGA AAAAAAAA                348
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1800 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GAATTCCGGG CCAAGAAGCC TAATGAGAAA AACAAACCAC TTGATAATAA GGGAGAAAAA     60
AGAAAAAGAA AAACTGAAGA AAAAGGCGTA GATAAAGATT TTGAGTCTTC TTCAATGAAA    120
ATCTCGAAAC TAGAAGTGAC TGAAATAGTG AAACCATCAC CAAAGCGCAA AATGGAACCT    180
GATACTGAAA AAATGGATAG GACCCCTGAA AAGGACAAAA TTTCTTTAAG TGCGCCAGCC    240
AAAAAAATCA AACTCAACAG AGAAACTGGG AAGAAAATTG GAAGTACAGA AAATATATCA    300
AACACAAAAG AACCCTCTGA AAAATTGGAG TCAACATCTA GCAAAGTTAA ACAAGAAAAA    360
GTCAAAGGAA AGGTCAGACG AAAAGTGACT GGAACTGAAG GATCCAGCTC AACTCTGGTG    420
GATTACACCA GTACGAGCTC AACTGGAGGC AGTCCTGTGC GGAAATCTGA AGAAAAAACA    480
GATACAAAGC GAACTGTGAT TAAAAGCATG GAAGAATATA ATAATGACAA TACCGCGCCA    540
CGTGAAGATG TTATCATTAT GATTCAGGTT CCTCAATCCA AATGGGATAA AGATGACTTT    600
GAATCTGAAG AAGAAGATGT TAAATCCACA CAGCCTATAT CAAGTGTAGG AAAACCTGCT    660
AGTGTTATAA AAAATGTTAG TACAAAGCCA TCAAATATAG TCAAGTATCC TGAGAAAGAA    720
AGTGAGCCAT CCGAGAAAAT TCAGAAATTC ACCAAGGACG TGAGCCATGA AATCATACAA    780
CATGAGGTTA AAAGTTCAAA AAACTCTGCA TCTAGTGAAA AAGGGAAAAC CAAAGATCGA    840
GATTATTCAG TGTTGGAAAA GGAGAACCCT GAAAAGAGGA AGAACAGCAC TCAGCCAGAG    900
AAAGAGAGTA ATTTGGACCG TCTGAATGAA CAAGGAAATT TTAAAAGTCT GTCTCAATCT    960
TCCAAAGAGG CTAGAACGTC AGATAAACAT GATTCCACTC GTGCTTCCTC AAATAAAGAC   1020
TTCACTCCCA ATAGAGACAA AAAAACTGAC TATGACACCA GAGAGTATTC AAGTTCCAAA   1080
CGTAGAGATG AAAAGAATGA ATTAACAAGA CGAAAAGACT CTCCTTCTCG GAATAAAGAT   1140
TCTGCATCTG GACAGAAAAA TAAACCAAGG GAAGAGAGAG ATTTGCCTAA AAAAGGAACA   1200
GGAGATTCCA AAAAAAGTAA TTCTAGTCCC TCAAGAGACA GAAAACCTCA TGATCACAAA   1260
GCCACTTATG ATACTAAACG GCCAAATGAA GAGACAAAAT CTGTAGATAA AAATCCTTGT   1320
AAGGATCGTG AGAAGCATGT ATTAGAAGCA AGGAACAATA AAGAGTCAAG TGGCAATAAA   1380
CTACTTTATA TACTTAACCC ACCAGAGACA CAGGTTGAAA AAGAGCAAAT TACTGGGCAA   1440
ATTGACAAGA GTACTGTCAA GCCTAAACCC CAGTTAAGTC ATTCCTCTAG ACTTTCCTCT   1500
GACTTAACTA GAGAAACTCA TGAAGCTGCT TTTGAACCAG ACTATAATGA AAGTGACAGT   1560
GAAAGTAATG TTTCTGTAAA AGAAGAGGAA TCTTCAGGAA ACATTTCTAA GGACCTGAAA   1620
GATAAAATAG TGGAGAAAGC AAAAGAGAGC CTGGACACAG CAGCAGTTGT CCAGGTGGGC   1680
ATAAGCAGGA ATCAGAGCCA CAGCAGCCCC AGCGTCAGCC CCAGCAGAAG CCACAGTCCT   1740
```

TCTGGAAGCC AGACCCGAAG CCACAGTAGC AGTGCCAGCT CAGCAGAAAG TCAGGACAGC 1800

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4868 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAATTCCGGC CGGAATTAAT TCCGGGGATT TCCTGGGGAA TCAGGAAGAT ATCCATAATC 60

TTCAACTGCG GGTAAAAGAG ACATCAAATG AGAATTTGAG ATTACTTCAT GTGATAGAGG 120

ACCGTGACAG AAAAGTTGAA AGTTTGCTAA ATGAAATGAA AGAATTAGAC TCAAAACTCC 180

ATTTACAGGA GGTACAACTA ATGACCAAAA TTGAAGCATG CATAGAATTG GAAAAAATAG 240

TTGGGGAACT TAAGAAAGAA AACTCAGATT TAAGTGAAAA ATTGGAATAT TTTTCTTGTG 300

ATCACCAGGA GTTACTCCAG AGAGTAGAAA CTTCTGAAGG CCTCAATTCT GATTTAGAAA 360

TGCATGCAGA TAAATCATCA CGTGAAGATA TTGGAGATAA TGTGGCCAAG GTGAATGACA 420

GCTGGAAGGA GAGATTTCTT GATGTGGAAA ATGAGCTGAG TAGGATCAGA TCGGAGAAAG 480

CTAGCATTGA GCATGAAGCC CTCTACCTGG AGGCTGACTT AGAGGTAGTT CAAACAGAGA 540

AGCTATGTTT AGAAAAAGAC AATGAAAATA AGCAGAAGGT TATTGTCTGC CTTGAAGAAG 600

AACTCTCAGT GGTCACAAGT GAGAGAAACC AGCTTCGTGG AGAATTAGAT ACTATGTCAA 660

AAAAAACCAC GGCACTGGAT CAGTTGTCTG AAAAAATGAA GGAGAAAACA CAAGAGCTTG 720

AGTCTCATCA AAGTGAGTGT CTCCATTGCA TTCAGGTGGC AGAGGCAGAG GTGAAGGAAA 780

AGACGGAACT CCTTCAGACT TTGTCCTCTG ATGTGAGTGA GCTGTTAAAA GACAAAACTC 840

ATCTCCAGGA AAAGCTGCAG AGTTTGGAAA AGGACTCACA GGCACTGTCT TTGACAAAAT 900

GTGAGCTGGA AAACCAAATT GCACAACTGA ATAAAGAGAA AGAATTGCTT GTCAAGGAAT 960

CTGAAAGCCT GCAGGCCAGA CTGAGTGAAT CAGATTATGA AAAGCTGAAT GTCTCCAAGG 1020

CCTTGGAGGC CGCACTGGTG GAGAAAGGTG AGTTCGCATT GAGGCTGAGC TCAACACAGG 1080

AGGAAGTGCA TCAGCTGAGA AGAGGCATCG AGAAACTGAG AGTTCGCATT GAGGCCGATG 1140

AAAAGAAGCA GCTGCACATC GCAGAGAAAC TGAAAGAACG CGAGCGGGAG AATGATTCAC 1200

TTAAGGTAAA AGTTGAGAAC CTTGAAAGGG AATTGCAGAT GTCAGAAGAA AACCAGGAGC 1260

TAGTGATTCT TGATGCCGAG AATTCCAAAG CAGAAGTAGA GACTCTAAAA ACACAAATAG 1320

AAGAGATGGC CAGAAGCCTG AAAGTTTTTG AATTAGACCT TGTCACGTTA AGGTCTGAAA 1380

AAGAAAATCT GACAAAACAA ATACAAGAAA AACAAGGTCA GTTGTCAGAA CTAGACAAGT 1440

TACTCTCTTC ATTTAAAAGT CTGTTAGAAG AAAAGGAGCA AGCAGAGATA CAGATCAAAG 1500

AAGAATCTAA AACTGCAGTG GAGATGCTTC AGAATCAGTT AAAGGAGCTA AATGAGGCAG 1560
TAGCAGCCTT GTGTGGTGAC CAAGAAATTA TGAAGGCCAC AGAACAGAGT CTAGACCCAC 1620
CAATAGAGGA AGAGCATCAG CTGAGAAATA GCATTGAAAA GCTGAGAGCC CGCCTAGAAG 1680
CTGATGAAAA GAAGCAGCTC TGTGTCTTAC AACAACTGAA GGAAAGTGAG CATCATGCAG 1740
ATTTACTTAA GGGTAGAGTG GAGAACCTTG AAAGAGAGCT AGAGATAGCC AGGACAAACC 1800
AAGAGCATGC AGCTCTTGAG GCAGAGAATT CCAAAGGAGA GGTAGAGACC CTAAAAGCAA 1860
AAATAGAAGG GATGACCCAA AGTCTGAGAG GTCTGGAATT AGATGTTGTT ACTATAAGGT 1920
CAGAAAAAGA AAATCTGACA AATGAATTAC AAAAAGAGCA AGAGCGAATA TCTGAATTAG 1980
AAATAATAAA TTCATCATTT GAAAATATTT TGCAAGAAAA AGAGCAAGAG AAAGTACAGA 2040
TGAAAGAAAA ATCAAGCACT GCCATGGAGA TGCTTCAAAC ACAATTAAAA GAGCTCAATG 2100
AGAGAGTGGC AGCCCTGCAT AATGACCAAG AAGCCTGTAA GGCCAAAGAG CAGAATCTTA 2160
GTAGTCAAGT AGAGTGTCTT GAACTTGAGA AGGCTCAGTT GCTACAAGGC CTTGATGAGG 2220
CCAAAAATAA TTATATTGTT TTGCAATCTT CAGTGAATGG CCTCATTCAA GAAGTAGAAG 2280
ATGGCAAGCA GAAACTGGAG AAGAAGGATG AAGAAATCAG TAGACTGAAA AATCAAATTC 2340
AAGACCAAGA GCAGCTTGTC TCTAAACTGT CCCAGGTGGA AGGAGAGCAC CAACTTTGGA 2400
AGGAGCAAAA CTTAGAACTG AGAAATCTGA CAGTGGAATT GGAGCAGAAG ATCCAAGTGC 2460
TACAATCCAA AAATGCCTCT TTGCAGGACA CATTAGAAGT GCTGCAGAGT TCTTACAAGA 2520
ATCTAGAGAA TGAGCTTGAA TTGACAAAAA TGGACAAAAT GTCCTTTGTT GAAAAAGTAA 2580
ACAAAAATGAC TGCAAAGGAA ACTGAGCTGC AGAGGGAAAT GCATGAGATG GCACAGAAAA 2640
CAGCAGAGCT GCAAGAAGAA CTCAGTGGAG AGAAAAATAG GCTAGCTGGA GAGTTGCAGT 2700
TACTGTTGGA AGAAATAAAG AGCAGCAAAG ATCAATTGAA GGAGCTCACA CTAGAAAATA 2760
GTGAATTGAA GAAGAGCCTA GATTGCATGC ACAAAGACCA GGTGGAAAAG GAAGGGAAAG 2820
TGAGAGAGGA AATAGCTGAA TATCAGCTAC GGCTTCATGA AGCTGAAAAG AAACACCAGG 2880
CTTTGCTTTT GGACACAAAC AAACAGTATG AAGTAGAAAT CCAGACATAC CGAGAGAAAT 2940
TGACTTCTAA AGAAGAATGT CTCAGTTCAC AGAAGCTGGA GATAGACCTT TTAAAGTCTA 3000
GTAAAGAAGA GCTCAATAAT TCATTGAAAG CTACTACTCA GATTTTGGAA GAATTGAAGA 3060
AAACCAAGAT GGACAATCTA AAATATGTAA ATCAGTTGAA GAAGGAAAAT GAACGTGCCC 3120
AGGGGAAAAT GAAGTTGTTG ATCAAATCCT GTAAACAGCT GGAAGAGGAA AAGGAGATAC 3180
TGCAGAAAGA ACTCTCTCAA CTTCAAGCTG CACAGGAGAA GCAGAAAACA GGTACTGTTA 3240
TGGATACCAA GGTCGATGAA TTAACAACTG AGATCAAAGA ACTGAAAGAA ACTCTTGAAG 3300
AAAAAACCAA GGAGGCAGAT GAATACTTGG ATAAGTACTG TTCCTTGCTT ATAAGCCATG 3360
AAAAGTTAGA GAAAGCTAAA GAGATGTTAG AGACACAAGT GGCCCATCTG TGTTCACAGC 3420
AATCTAAACA AGATTCCCGA GGGTCTCCTT TGCTAGGTCC AGTTGTTCCA GGACCATCTC 3480
CAATCCCTTC TGTTACTGAA AAGAGGTTAT CATCTGGCCA AAATAAAGCT TCAGGCAAGA 3540

```
GGCAAAGATC CAGTGGAATA TGGGAGAATG GTGGAGGACC AACACCTGCT ACCCCAGAGA   3600
CCTTTTCTAA AAAAAGCAAG AAAGCAGTCA TGAGTGGTAT TCACCCTGCA GAAGACACGG   3660
AAGGTACTGA GTTTGAGCCA GAGGGACTTC CAGAAGTTGT AAAGAAAGGG TTTGCTGACA   3720
TCCCGACAGG AAAGACTAGC CCATATATCC TGCGAAGAAC AACCATGGCA ACTGGGAGCA   3780
GGCCCGGCCT GGCTGCACAC AAGTTACCCC TATCCCCACT GACTGTCCCC AAACAAAATC   3840
TTGCAGAGTC CTCCAAACCA ACAGCTGGTG GCAGCAGATC ACAAAAGGTG AAAGTTGCTC   3900
AGCGGAGCCC AGTAGATTCA GGCACCATCC TCCGAGAACC CACCACGAAA TCCGTCCCAG   3960
TCAATAATCT TCCTGAGAGA AGTCCGACTG ACAGCCCCAG AGAGGGCCTG AGGGTCAAGC   4020
GCCGGCGACT TGTCCCCAGC CCCAAAGCTG GACTGGAGTC CAAGGGCAGT GAGAACTGTA   4080
AGGTCCAGTG AAGGCACTTT GTGTGTCAGT ACCCCTGGGA GGTGCCAGTC ATTGAATAGA   4140
TAAGGCTGTG CCTACAGGAC TTCTCTTTAG TCAGGGCATG CTTTATTAGT GAGGAGAAAA   4200
CAATTCCTTA GAAGTCTTAA ATATATTGTA CTCTTTAGAT CTCCCATGTG TAGGTATTGA   4260
AAAAGTTTGG AAGCACTGAT CACCTGTTAG CATTGCAATT CCTCTACTGC AATGTAAATA   4320
GTATAAAGCT ATGTATATAA AGCTTTTTGG TAATATGTTA CAATTAAAAT GACAAGCACT   4380
ATATCACAAT CTCTGTTTGT ATGTGGGTTT TACACTAAAA AAATGCAAAA CACATTTTAT   4440
TCTTCTAATT AACAGCTCCT AGGAAAATGT AGACTTTTGC TTTATGATAT TCTATCTGTA   4500
GTATGAGGCA TGGAATAGTT TTGTATCGGG AATTTCTCAG AGCTGAGTAA AATGAAGGAA   4560
AAGCATGTTA TGTGTTTTTA AGGAAAATGT GCACACATAT ACATGTAGGA GTGTTTATCT   4620
TTCTCTTACA ATCTGTTTTA GACATCTTTG CTTATGAAAC CTGTACATAT GTGTGTGTGG   4680
GTATGTGTTT ATTTCCAGTG AGGGCTGCAG GCTTCCTAGA GGTGTGCTAT ACCATGCGTC   4740
TGTCGTTGTG CTTTTTTCTG TTTTTAGACC AATTTTTTAC AGTTCTTTGG TAAGCATTGT   4800
CGTATCTGGT GATGGATTAA CATATAGCCT TTGTTTTCTA ATAAAATAGT CGCCTTCGTA   4860
AAAAAAAA                                                            4868
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2492 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1428

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
CTT TGC AGG CAG CGG CGG CCG GGG GCG GAG CGG GAT CGA GCC CTC GCC      48
Leu Cys Arg Gln Arg Arg Pro Gly Ala Glu Arg Asp Arg Ala Leu Ala
 1               5                  10                  15
```

```
GAG GCC TGC CGC CAT GGG CCC GCG CCG CCG CCG CCG CCT GTC ACC CGG      96
Glu Ala Cys Arg His Gly Pro Ala Pro Pro Pro Pro Pro Val Thr Arg
             20                  25                  30

GCC GCG CGG GCC GTG AGC GTC ATG GCC TTG GCC GGG GCC CCT GCG GGC     144
Ala Ala Arg Ala Val Ser Val Met Ala Leu Ala Gly Ala Pro Ala Gly
         35                  40                  45

GGC CCA TGC GCG CCG GCG CTG GAG GCC CTG CTC GGG GCC GGC GCG CTG     192
Gly Pro Cys Ala Pro Ala Leu Glu Ala Leu Leu Gly Ala Gly Ala Leu
     50                  55                  60

CGG CTG CTC GAC TCC TCG CAG ATC GTC ATC ATC TCC GCC GCG CAG GAC     240
Arg Leu Leu Asp Ser Ser Gln Ile Val Ile Ile Ser Ala Ala Gln Asp
 65                  70                  75                  80

GCC AGC GCC CCG CCG GCT CCC ACC GGC CCC GCG GCG CCC GCC GCC GGC     288
Ala Ser Ala Pro Pro Ala Pro Thr Gly Pro Ala Ala Pro Ala Ala Gly
                 85                  90                  95

CCC TGC GAC CCT GAC CTG CTG CTC TTC GCC ACA CCG CAG GCG CCC CGG     336
Pro Cys Asp Pro Asp Leu Leu Leu Phe Ala Thr Pro Gln Ala Pro Arg
            100                 105                 110

CCC ACA CCC AGT GCG CCG CGG CCC GCG CTC GGC CGC CCG CCG GTG AAG     384
Pro Thr Pro Ser Ala Pro Arg Pro Ala Leu Gly Arg Pro Pro Val Lys
        115                 120                 125

CGG AGG CTG GAC CTG GAA ACT GAC CAT CAG TAC CTG GCC GAG AGC AGT     432
Arg Arg Leu Asp Leu Glu Thr Asp His Gln Tyr Leu Ala Glu Ser Ser
    130                 135                 140

GGG CCA GCT CGG GGC AGA GGC CGC CAT CCA GGA AAA GGT GTG AAA TCC     480
Gly Pro Ala Arg Gly Arg Gly Arg His Pro Gly Lys Gly Val Lys Ser
145                 150                 155                 160

CCG GGG GAG AAG TCA CGC TAT GAG ACC TCA CTG AAT CTG ACC ACC AAG     528
Pro Gly Glu Lys Ser Arg Tyr Glu Thr Ser Leu Asn Leu Thr Thr Lys
                165                 170                 175

CGC TTC CTG GAG CTG CTG AGC CAC TCG GCT GAC GGT GTC GTC GAC CTG     576
Arg Phe Leu Glu Leu Leu Ser His Ser Ala Asp Gly Val Val Asp Leu
            180                 185                 190

AAC TGG GCT GCC GAG GTG CTG AAG GTG CAG AAG CGG CGC ATC TAT GAC     624
Asn Trp Ala Ala Glu Val Leu Lys Val Gln Lys Arg Arg Ile Tyr Asp
        195                 200                 205

ATC ACC AAC GTC CTT GAG GGC ATC CAG CTC ATT GCC AAG AAG TCC AAG     672
Ile Thr Asn Val Leu Glu Gly Ile Gln Leu Ile Ala Lys Lys Ser Lys
    210                 215                 220

AAC CAC ATC CAG TGG CTG GGC AGC CAC ACC ACA GTG GGC GTC GGC GGA     720
Asn His Ile Gln Trp Leu Gly Ser His Thr Thr Val Gly Val Gly Gly
225                 230                 235                 240

CGG CTT GAG GGG TTG ACC CAG GAC CTC CGA CAG CTG CAG GAG AGC GAG     768
Arg Leu Glu Gly Leu Thr Gln Asp Leu Arg Gln Leu Gln Glu Ser Glu
                245                 250                 255

CAG CAG CTG GAC CAC CTG ATG AAT ATC TGT ACT ACG CAG CTG CGC CTG     816
Gln Gln Leu Asp His Leu Met Asn Ile Cys Thr Thr Gln Leu Arg Leu
            260                 265                 270

CTC TCC GAG GAC ACT GAC AGC CAG CGC CTG GCC TAC GTG ACG TGT CAG     864
Leu Ser Glu Asp Thr Asp Ser Gln Arg Leu Ala Tyr Val Thr Cys Gln
        275                 280                 285
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GAC CTT CGT AGC ATT GCA GAC CCT GCA GAG CAG ATG GTT ATG GTG ATC      912
Asp Leu Arg Ser Ile Ala Asp Pro Ala Glu Gln Met Val Met Val Ile
    290                 295                 300

AAA GCC CCT CCT GAG ACC CAG CTC CAA GCC GTG GAC TCT TCG GAG AAC      960
Lys Ala Pro Pro Glu Thr Gln Leu Gln Ala Val Asp Ser Ser Glu Asn
305                 310                 315                 320

TTT CAG ATC TCC CTT AAG AGC AAA CAA GGC CCG ATC GAT GTT TTC CTG     1008
Phe Gln Ile Ser Leu Lys Ser Lys Gln Gly Pro Ile Asp Val Phe Leu
                325                 330                 335

TGC CCT GAG GAG ACC GTA GGT GGG ATC AGC CCT GGG AAG ACC CCA TCC     1056
Cys Pro Glu Glu Thr Val Gly Gly Ile Ser Pro Gly Lys Thr Pro Ser
            340                 345                 350

CAG GAG GTC ACT TCT GAG GAG GAG AAC AGG GCC ACT GAC TCT GCC ACC     1104
Gln Glu Val Thr Ser Glu Glu Glu Asn Arg Ala Thr Asp Ser Ala Thr
        355                 360                 365

ATA GTG TCA CCA CCA CCA TCA TCT CCC CCC TCA TCC CTC ACC ACA GAT     1152
Ile Val Ser Pro Pro Pro Ser Ser Pro Pro Ser Ser Leu Thr Thr Asp
    370                 375                 380

CCC AGC CAG TCT CTA CTC AGC CTG GAG CAA GAA CCG CTG TTG TCC CGG     1200
Pro Ser Gln Ser Leu Leu Ser Leu Glu Gln Glu Pro Leu Leu Ser Arg
385                 390                 395                 400

ATG GGC AGC CTG CGG GCT CCC GTG GAC GAG GAC CGC CTG TCC CCG CTG     1248
Met Gly Ser Leu Arg Ala Pro Val Asp Glu Asp Arg Leu Ser Pro Leu
                405                 410                 415

GTG GCG GCC GAC TCG CTC CTG GAG CAT GTG CGG GAG GAC TTC TCC GGC     1296
Val Ala Ala Asp Ser Leu Leu Glu His Val Arg Glu Asp Phe Ser Gly
            420                 425                 430

CTC CTC CCT GAG GAG TTC ATC AGC CTT TCC CCA CCC CAC GAG GCC CTC     1344
Leu Leu Pro Glu Glu Phe Ile Ser Leu Ser Pro Pro His Glu Ala Leu
        435                 440                 445

GAC TAC CAC TTC GGC CTC GAG GAG GGC GAG GGC ATC AGA GAC CTC TTC     1392
Asp Tyr His Phe Gly Leu Glu Glu Gly Glu Gly Ile Arg Asp Leu Phe
    450                 455                 460

GAC TGT GAC TTT GGG GAC CTC ACC CCC CTG GAT TTC TGACAGGGCT          1438
Asp Cys Asp Phe Gly Asp Leu Thr Pro Leu Asp Phe
465                 470                 475

TGGAGGGACC AGGGTTTCCA GAGATGCTCA CCTTGTCTCT GCAGCCCTGG AGCCCCCTGT   1498

CCCTGGCCGT CCTCCCAGCC TGTTTGGAAA CATTTAATTT ATACCCCTCT CCTCTGTCTC   1558

CAGAAGCTTC TAGCTCTGGG GTCTGGCTAC CGCTAGGAGG CTGAGCAAGC CAGGAAGGGA   1618

AGGAGTCTGT GTGGTGTGTA TGTGCATGCA GCCTACACCC ACACGTGTGT ACCGGGGGTG   1678

AATGTGTGTG AGCATGTGTG TGTGCATGTA CCGGGGAATG AAGGTGAACA TACACCTCTG   1738

TGTGTGCACT GCAGACACGC CCCAGTGTGT CCACATGTGT GTGCATGAGT CCATGTGTGC   1798

GCGTGGGGGG GCTCTAACTG CACTTTCGGC CCTTTTGCTC TGGGGGTCCC ACAAGGCCCA   1858

GGGCAGTGCC TGCTCCCAGA ATCTGGTGCT CTGACCAGGC CAGGTGGGGA GGCTTTGGCT   1918

GGCTGGGCGT GTAGGACGGT GAGAGCACTT CTGTCTTAAA GGTTTTTTCT GATTGAAGCT   1978

TTAATGGAGC GTTATTTATT TATCGAGGCC TCTTTGGTGA GCCTGGGGAA TCAGCAAAGG   2038
```

GGAGGAGGGG TGTGGGGTTG ATACCCCAAC TCCCTCTACC CTTGAGCAAG GGCAGGGGTC 2098

CCTGAGCTGT TCTTCTGCCC CATACTGAAG GAACTGAGGC CTGGGTGATT TATTTATTGG 2158

GAAAGTGAGG GAGGGAGACA GACTGACTGA CAGCCATGGG TGGTCAGATG GTGGGGTGGG 2218

CCCTCTCCAG GGGGCCAGTT CAGGGCCCCA GCTGCCCCCC AGGATGGATA TGAGATGGGA 2278

GAGGTGAGTG GGGGACCTTC ACTGATGTGG GCAGGAGGGG TGGTGAAGGC CTCCCCCAGC 2338

CCAGACCCTG TGGTCCCTCC TGCAGTGTCT GAAGCGCCTG CCTCCCCACT GCTCTGCCCC 2398

ACCCTCCAAT CTGCACTTTG ATTTGCTTCC TAACAGCTCT GTTCCCTCCT GCTTTGGTTT 2458

TAATAAATAT TTTGATGACG TTAAAAAAAA AAAA 2492

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 476 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Leu Cys Arg Gln Arg Arg Pro Gly Ala Glu Arg Asp Arg Ala Leu Ala
 1               5                  10                  15

Glu Ala Cys Arg His Gly Pro Ala Pro Pro Pro Pro Pro Val Thr Arg
            20                  25                  30

Ala Ala Arg Ala Val Ser Val Met Ala Leu Ala Gly Ala Pro Ala Gly
        35                  40                  45

Gly Pro Cys Ala Pro Ala Leu Glu Ala Leu Leu Gly Ala Gly Ala Leu
    50                  55                  60

Arg Leu Leu Asp Ser Ser Gln Ile Val Ile Ile Ser Ala Ala Gln Asp
65                  70                  75                  80

Ala Ser Ala Pro Pro Ala Pro Thr Gly Pro Ala Ala Pro Ala Ala Gly
                85                  90                  95

Pro Cys Asp Pro Asp Leu Leu Leu Phe Ala Thr Pro Gln Ala Pro Arg
            100                 105                 110

Pro Thr Pro Ser Ala Pro Arg Pro Ala Leu Gly Arg Pro Pro Val Lys
        115                 120                 125

Arg Arg Leu Asp Leu Glu Thr Asp His Gln Tyr Leu Ala Glu Ser Ser
    130                 135                 140

Gly Pro Ala Arg Gly Arg Gly Arg His Pro Gly Lys Gly Val Lys Ser
145                 150                 155                 160

Pro Gly Glu Lys Ser Arg Tyr Glu Thr Ser Leu Asn Leu Thr Thr Lys
                165                 170                 175

Arg Phe Leu Glu Leu Leu Ser His Ser Ala Asp Gly Val Val Asp Leu
            180                 185                 190

Asn Trp Ala Ala Glu Val Leu Lys Val Gln Lys Arg Arg Ile Tyr Asp
        195                 200                 205
```

Ile Thr Asn Val Leu Glu Gly Ile Gln Leu Ile Ala Lys Lys Ser Lys
210 215 220

Asn His Ile Gln Trp Leu Gly Ser His Thr Thr Val Gly Val Gly Gly
225 230 235 240

Arg Leu Glu Gly Leu Thr Gln Asp Leu Arg Gln Leu Gln Glu Ser Glu
245 250 255

Gln Gln Leu Asp His Leu Met Asn Ile Cys Thr Thr Gln Leu Arg Leu
260 265 270

Leu Ser Glu Asp Thr Asp Ser Gln Arg Leu Ala Tyr Val Thr Cys Gln
275 280 285

Asp Leu Arg Ser Ile Ala Asp Pro Ala Glu Gln Met Val Met Val Ile
290 295 300

Lys Ala Pro Pro Glu Thr Gln Leu Gln Ala Val Asp Ser Ser Glu Asn
305 310 315 320

Phe Gln Ile Ser Leu Lys Ser Lys Gln Gly Pro Ile Asp Val Phe Leu
325 330 335

Cys Pro Glu Glu Thr Val Gly Gly Ile Ser Pro Gly Lys Thr Pro Ser
340 345 350

Gln Glu Val Thr Ser Glu Glu Glu Asn Arg Ala Thr Asp Ser Ala Thr
355 360 365

Ile Val Ser Pro Pro Pro Ser Ser Pro Pro Ser Ser Leu Thr Thr Asp
370 375 380

Pro Ser Gln Ser Leu Leu Ser Leu Glu Gln Glu Pro Leu Leu Ser Arg
385 390 395 400

Met Gly Ser Leu Arg Ala Pro Val Asp Glu Asp Arg Leu Ser Pro Leu
405 410 415

Val Ala Ala Asp Ser Leu Leu Glu His Val Arg Glu Asp Phe Ser Gly
420 425 430

Leu Leu Pro Glu Glu Phe Ile Ser Leu Ser Pro Pro His Glu Ala Leu
435 440 445

Asp Tyr His Phe Gly Leu Glu Glu Gly Glu Gly Ile Arg Asp Leu Phe
450 455 460

Asp Cys Asp Phe Gly Asp Leu Thr Pro Leu Asp Phe
465 470 475

WE CLAIM:

1. An isolated nucleic acid molecule ncoding a retinoblastoma-associated polypeptide.

2. The isolated nucleic acid molecule of claim 1, wherein the encoded retinoblastoma-associated polypeptide has transcriptional factor E2F biological activity.

3. The isolated nucleic acid molecule of claim 1, wherein the encoded retinoblastoma-associated polypeptide has RB-binding activity.

4. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule is a DNA molecule, a cDNA molecule or an RNA molecule.

5. An isolated nucleic acid molecule that hybridizes under stringent conditions to the isolated nucleic acid molecule of claim 1.

6. An isolated and purified polypeptide encoded by the nucleic acid molecule of claim 1.

7. An isolated and purified polypeptide encoded by the nucleic acid molecule of claim 2.

8. An isolated and purified polypeptide encoded by the nucleic acid molecule of claim 3.

9. A vector comprising the isolated nucleic acid molecule of claim 1.

10. A plasmid comprising the vector of claim 9.

11. A virus comprising the vector of claim 9.

12. A host cell comprising the vector of claim 9.

13. The host c ll of claim 12, wherein the host cell is a bacterium, a yeast cell or a mammalian cell.

14. An antibody capable of specifically binding to a retinoblastoma-associated polypeptide present in the nucleus of the cell.

15. An immunologically reactive polypeptide fragment of the antibody of claim 14.

16. The antibody of claim 14, wherein the antibody is a monoclonal antibody.

17. The antibody of claim 14, wherein said antibody is labelled with a detectable marker.

18. A hybridoma cell line producing the antibody of claim 17.

19. A method for detecting a retinoblastoma-associated protein in a sample comprising: a. contacting the antibody of claim 14 with the sample under conditions permitting formation of an antibody-antigen complex; b. detecting the presence of any complex so formed; c. the presence of complex indicating the presence of retinoblastoma-associated protein in the sample.

20. A method of recombinantly producing a retinoblastoma-associated protein which comprises growing the host cell of claim 12 under suitable conditions permitting production of the protein and recovering and purifying the resulting protein so produced.

21. Th recombinantly produced protein of claim 20.

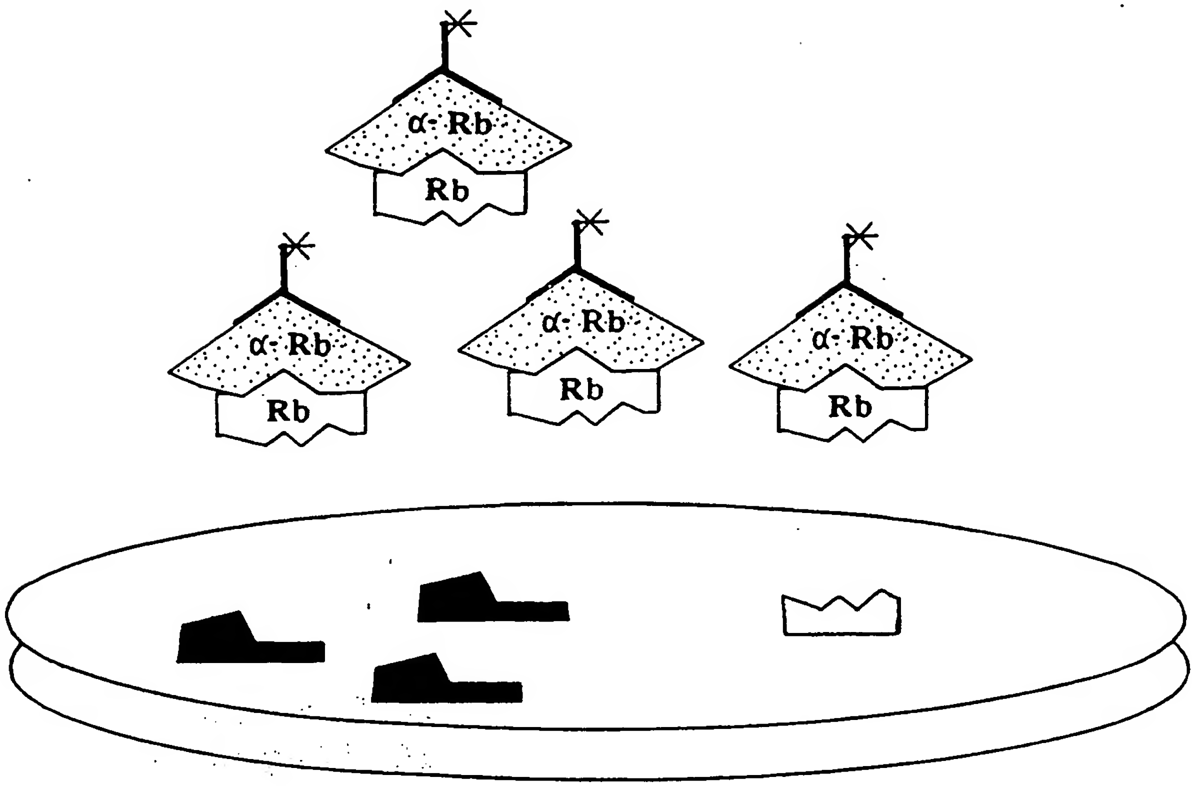


FIG. 1A

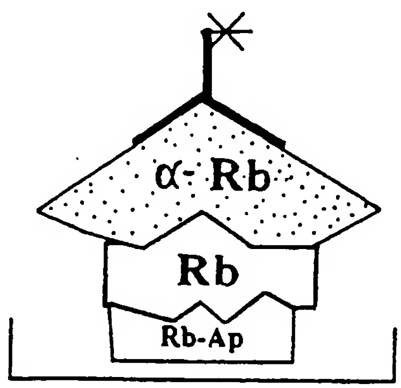


FIG. 1B

Rb-Ap

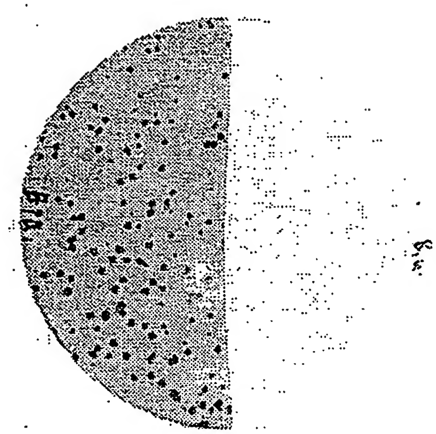


FIG. 1C

T antigen

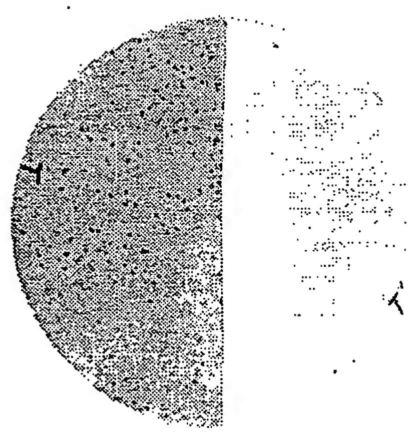


FIG. 1D

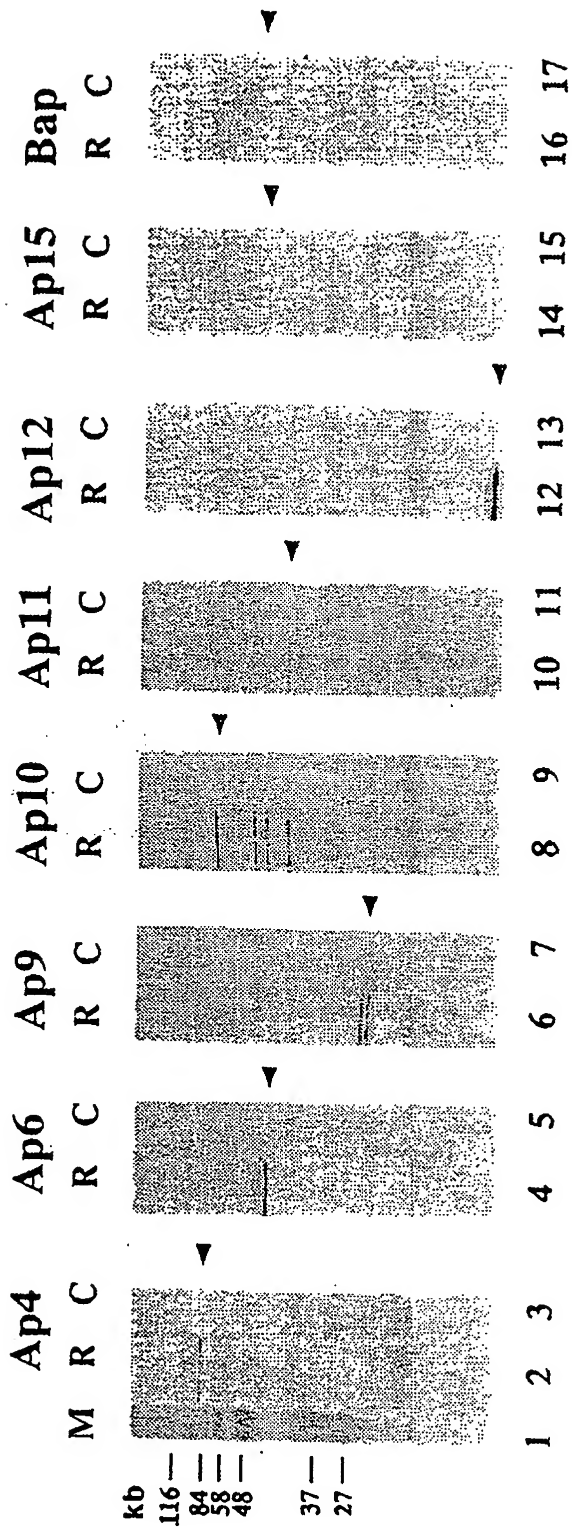

Ap4
M R C
Ap6
R C
Ap9
R C
Ap10
R C
Ap11
R C
Ap12
R C
Ap15
R C
Bap
R C
kb
116
84
58
48
37
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

FIG. 2

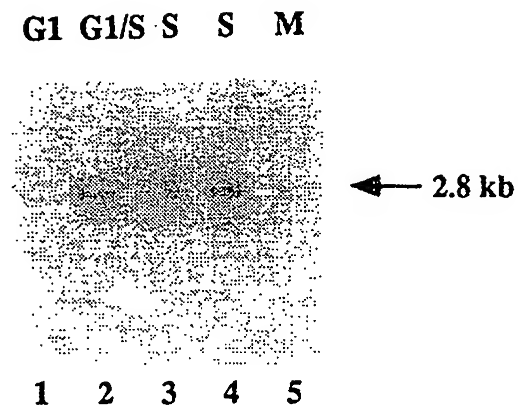

G1 G1/S S S M
2.8 kb
1 2 3 4 5

FIG. 3

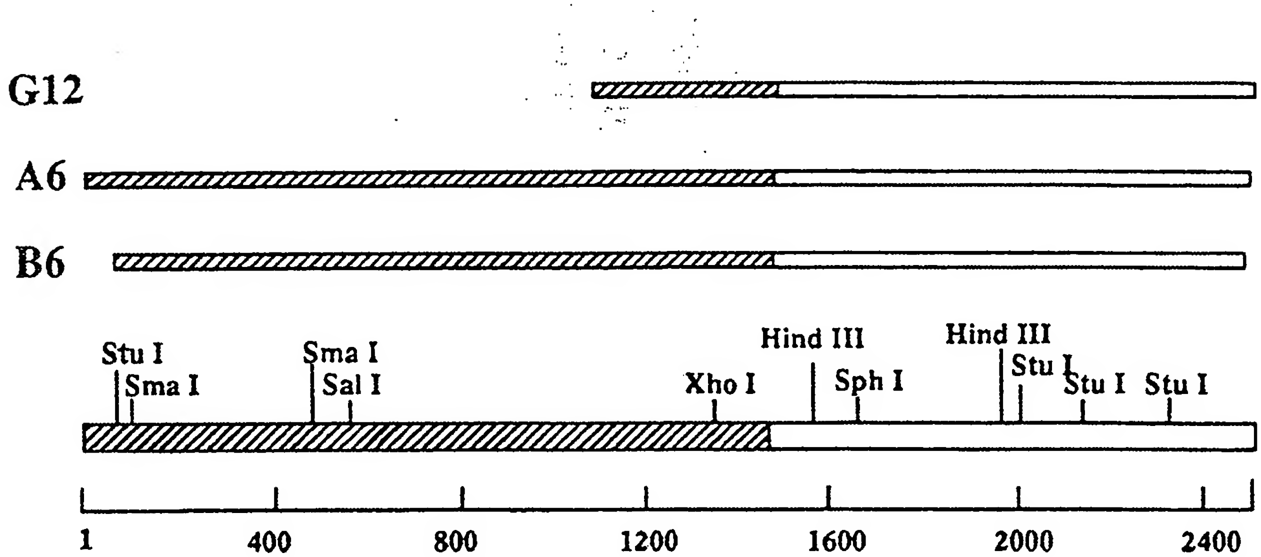

G12
A6
B6
Stu I
Sma I
Sma I
Sal I
Xho I
Hind III
Sph I
Hind III
Stu I
Stu I
Stu I
1
400
800
1200
1600
2000
2400

FIG. 4A

CTTTGCAGGCAGCGGCGGCCGGGGGCGGAGCGGGATCGAGCCCTCGCCGAGGCCTGCCGCCATGGGCCCGCGCCGCCGCCGCCGCCTGTC 90
L C R Q R R P G A E R D R A L A E A C R H G P A P P P P P V
ACCCGGGCCGCGCGGGCCGTGAGCGTCATGGCCTTGGCCGGGGCCCCTGCGGGCGGCCCATGCGCGCCGGCGCTGGAGGCCCTGCTCGGG 180
T R A A R A V S V M A L A G A P A G G P C A P A L E A L L G
GCCGGCGCGCTGCGGCTGCTCGACTCCTCGCAGATCGTCATCATCTCCGCCGCGCAGGACGCCAGCGCCCCGCCGGCTCCCACCGGCCCC 270
A G A L R L L D S S Q I V I I S A A Q D A S A P P A P T G P
GCGGCGCCCGCCGCCGGCCCCTGCGACCCTGACCTGCTGCTCTTCGCCACACCGCAGGCGCCCCGGCCCACACCCAGTGCGCCGCGGCCC 360
A A P A A G P C D P D L L L F A T P Q A P R P T P S A P R P
GCGCTCGGCCGCCCGCCGGTGAAGCGGAGGCTGGACCTGGAAACTGACCATCAGTACCTGGCCGAGAGCAGTGGGCCAGCTCGGGGCAGA 450
A L G R P P V K R R L D L E T D H Q Y L A E S S G P A R G R
GGCCGCCATCCAGGAAAAGGTGTGAAATCCCCGGGGGAGAAGTCACGCTATGAGACCTCACTGAATCTGACCACCAAGCGCTTCCTGGAG 540
G R H P G K G V K S P G E K S R Y E T S L N L T T K R F L E
CTGCTGAGCCACTCGGCTGACGGTGTCGTCGACCTGAACTGGGCTGCCGAGGTGCTGAAGGTGCAGAAGCGGCGCATCTATGACATCACC 630
L L S H S A D G V V D $\boxed{L}$ N W A A E V $\boxed{L}$ K V Q K R R $\boxed{I}$ Y D I T
AACGTCCTTGAGGGCATCCAGCTCATTGCCAAGAAGTCCAAGAACCACATCCAGTGGCTGGGCAGCCACACCACAGTGGGCGTCGGCGGA 720
N V $\boxed{L}$ E G I Q L I A K K S K N H I Q W L G S H T T V G V G G
CGGCTTGAGGGGTTGACCCAGGACCTCCGACAGCTGCAGGAGAGCGAGCAGCAGCTGGACCACCTGATGAATATCTGTACTACGCAGCTG 810
R L E G L T Q D L R Q L Q E S E Q Q L D H L M N I C T T Q L
CGCCTGCTCTCCGAGGACACTGACAGCCAGCGCCTGGCCTACGTGACGTGTCAGGACCTTCGTAGCATTGCAGACCCTGCAGAGCAGATG 900
R L L S E D T D S Q R L A Y V T C Q D L R S I A D P A E Q M
GTTATGGTGATCAAAGCCCCTCCTGAGACCCAGCTCCAAGCCGTGGACTCTTCGGAGAACTTTCAGATCTCCCTTAAGAGCAAACAAGGC 990
V M V I K A P P E T Q L Q A V D S S E N F Q I S L K S K Q G

FIG. 4B-1

```
CCGATCGATGTTTTCCTGTGCCCTGAGGAGACCGTAGGTGGGATCAGCCCTGGGAAGACCCCATCCCAGGAGGTCAATTCTGAGGAGGAG   1080
 P  I  D  V  F  L  C  P  E  E  T  V  G  G  I  S  P  G  K  T  P  S  Q  E  V  T  S  E  E  E
AACAGGGCCACTGACTCTGCCACCATAGTGTCACCACCACCATCATCTCCCCCCTCATCCCTCACCACAGATCCCAGCCAGTCTCTACTC   1170
 N  R  A  T  D  S  A  T  I  V  S  P  P  P  S  S  P  P  S  S  L  T  T  D  P  S  Q  S  L  L
AGCCTGGAGCAAGAACCGCTGTTGTCCCGGATGGGCAGCCTGCGGGCTCCCGTGGACGAGGACCGCCTGTCCCCGCTGGTGGCGGCCGAC   1260
 S  L  E  Q  E  P  L  L  S  R  M  G  S  L  R  A  P  V  D  E  D  R  L  S  P  L  V  A  A  D
TCGCTCCTGGAGCATGTGCGGGAGGACTTCTCCGGCCTCCTCCCTGAGGAGTTCATCAGCCTTTCCCCACCCCACGAGGCCCTCGACTAC   1350
 S  L  L  E  H  V  R  E  D  F  S  G  L  L  P  E  E  F  I  S  L  S  P  P  H  E  A  L  D  Y
CACTTCGGCCTCGAGGAGGGCGAGGGCATCAGAGACCTCTTCGACTGTGACTTTGGGGACCTCACCCCCCTGGATTTCTGACAGGGCTTG   1440
 H  F  G  L  E  E  G  E  G  I  R  D  L  F  D  C  D  F  G  D  L  T  P  L  D  F
GAGGGACCAGGGTTTCCAGAGATGCTCACCTTGTCTCTGCAGCCCTGGAGCCCCCTGTCCCTGGCCGTCCTCCCAGCCTGTTTGGAAACA   1530
TTTAATTTATACCCCTCTCCTCTGTCTCCAGAAGCTTCTAGCTCTGGGGTCTGGCTACCGCTAGGAGGCTGAGCAAGCCAGGAAGGGAAG
GAGTCTGTGTGGTGTGTATGTGCATGCAGCCTACACCCACACGTGTGTACCGGGGGTGAATGTGTGTGAGCATGTGTGTGTGCATGTACC
GGGGAATGAAGGTGAACATACACCTCTGTGTGTGCACTGCAGACACGCCCCAGTGTGTCCACATGTGTGTGCATGAGTCCATGTGTGCGC
GTGGGGGGGCTCTAACTGCACTTTCGGCCCTTTTGCTCTGGGGGTCCCACAAGGCCCAGGGCAGTGCCTGCTCCCAGAATCTGGTGCTCT
GACCAGGCCAGGTGGGGAGGCTTTGGCTGGCTGGGCGTGTAGGACGGTGAGAGCACTTCTGTCTTAAAGGTTTTTTCTGATTGAAGCTTT
AATGGAGCGTTATTTATTTATCGAGGCCTCTTTGGTGAGCCTGGGGAATCAGCAAAGGGGAGGAGGGGTGTGGGGTTGATACCCCAACTC
CCTCTACCCTTGAGCAAGGGCAGGGGTCCCTGAGCTGTTCTTCTGCCCCATACTGAAGGAACTGAGGCCTGGGTGATTTATTTATTGGGA
AAGTGAGGGAGGGAGACAGACTGACTGACAGCCATGGGTGGTCAGATGGTGGGGTGGGCCCTCTCCAGGGGGCCAGTTCAGGGCCCCAGC
TGCCCCCCAGGATGGATATGAGATGGGAGAGGTGAGTGGGGGACCTTCACTGATGTGGGCAGGAGGGGTGGTGAAGGCCTCCCCCAGCCC
AGACCCTGTGGTCCCTCCTGCAGTGTCTGAAGCGCCTGCCTCCCCACTGCTCTGCCCCACCCTCCAATCTGCACTTTGATTTGCTTCCTA
ACAGCTCTGTTCCCTCCTGCTTTGGTTTTAATAAATATTTTGATGACGTTAAAAAAAAAAAA   2492
```

FIG. 4B-2

FIG. 5A

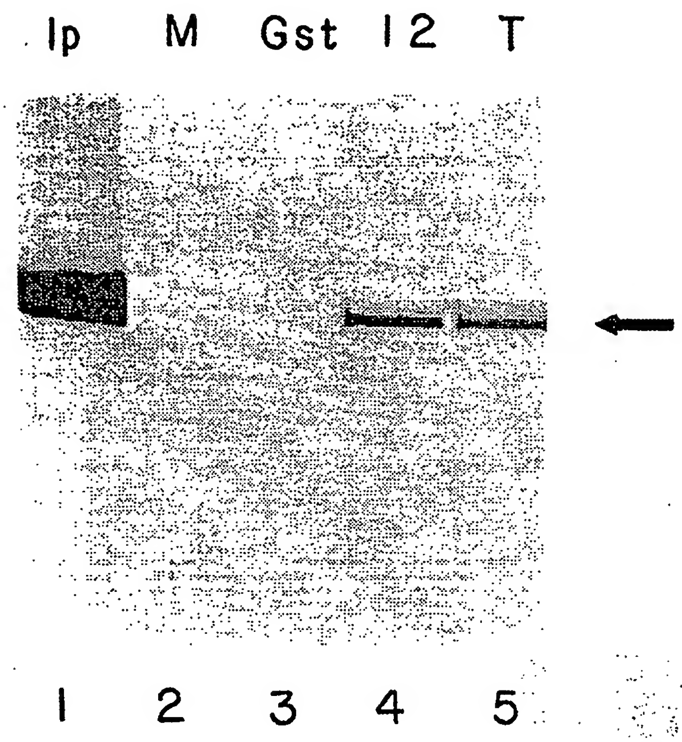

Ip
M
Gst
12
T
1
2
3
4
5

FIG. 5B

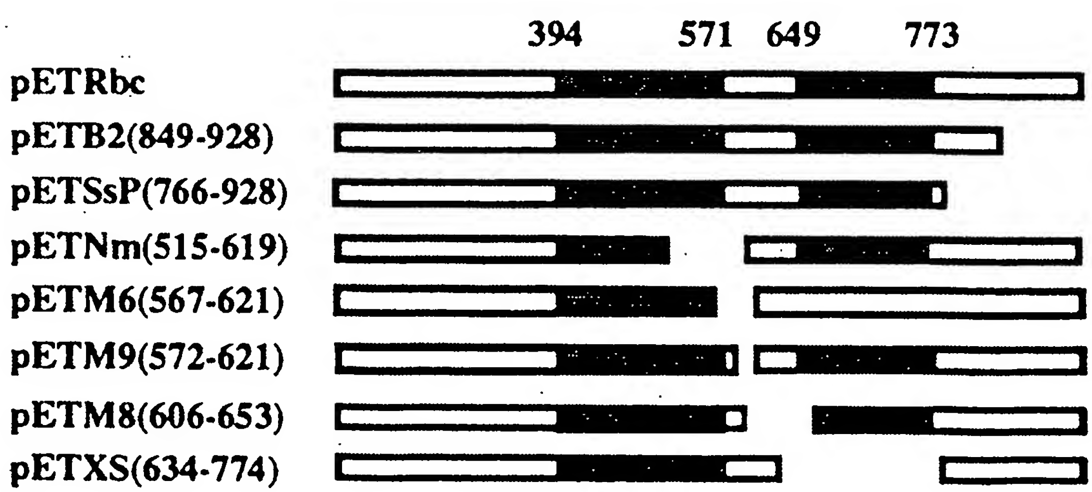

394
571
649
773
pETRbc
pETB2(849-928)
pETSsP(766-928)
pETNm(515-619)
pETM6(567-621)
pETM9(572-621)
pETM8(606-653)
pETXS(634-774)

FIG. 5C

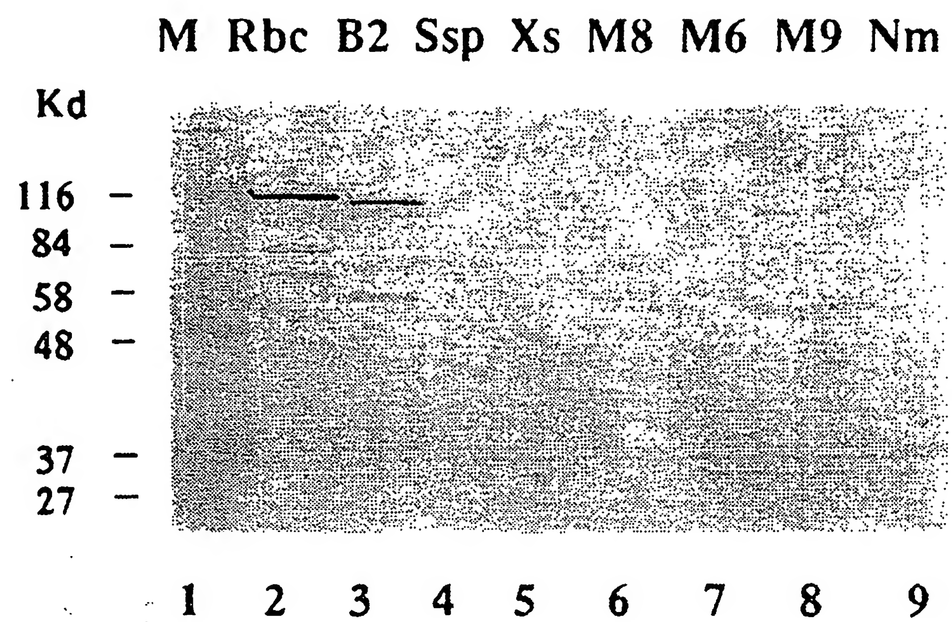

M Rbc B2 Ssp Xs M8 M6 M9 Nm
Kd
116
84
58
48
37
27
1 2 3 4 5 6 7 8 9

FIG. 5D

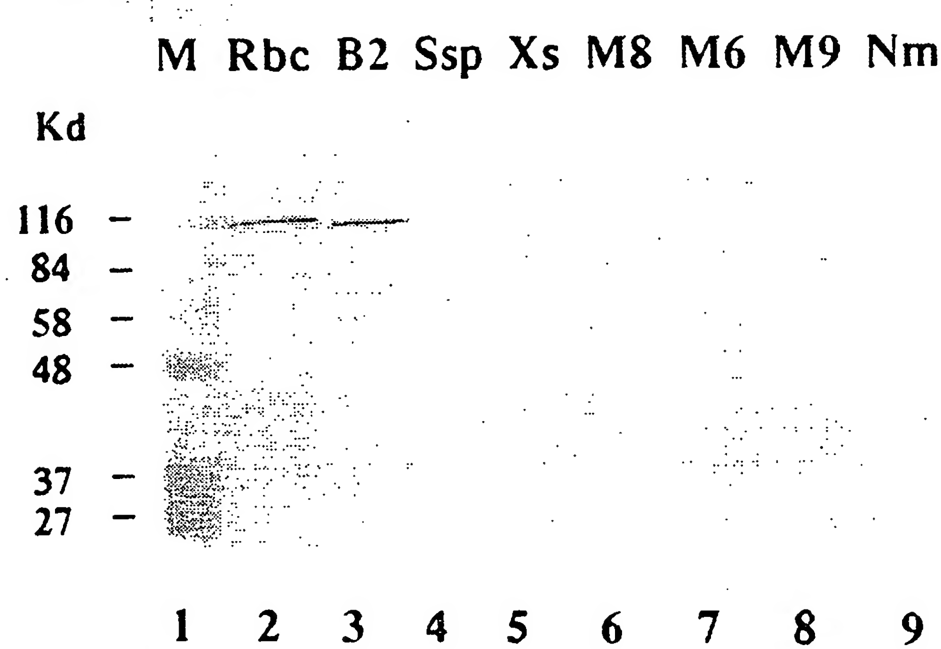
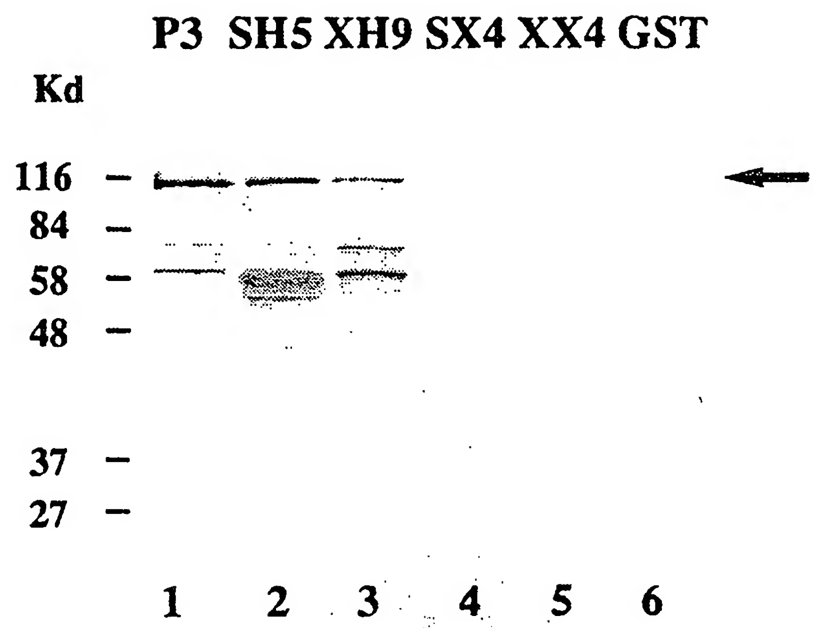

M Rbc B2 Ssp Xs M8 M6 M9 Nm
Kd
116
84
58
48
37
27
1 2 3 4 5 6 7 8 9

FIG. 6A


P3 SH5 XH9 SX4 XX4 GST
Kd
116
84
58
48
37
27
1 2 3 4 5 6

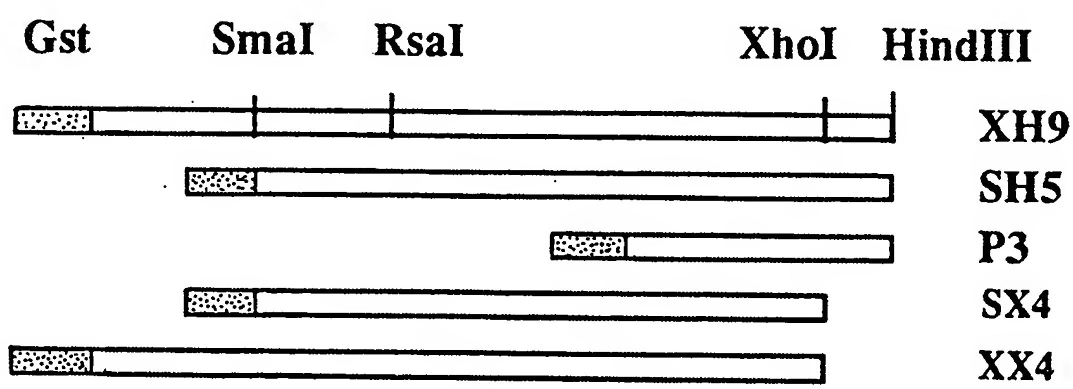

Gst
SmaI
RsaI
XhoI
HindIII
XH9
SH5
P3
SX4
XX4

FIG. 6B

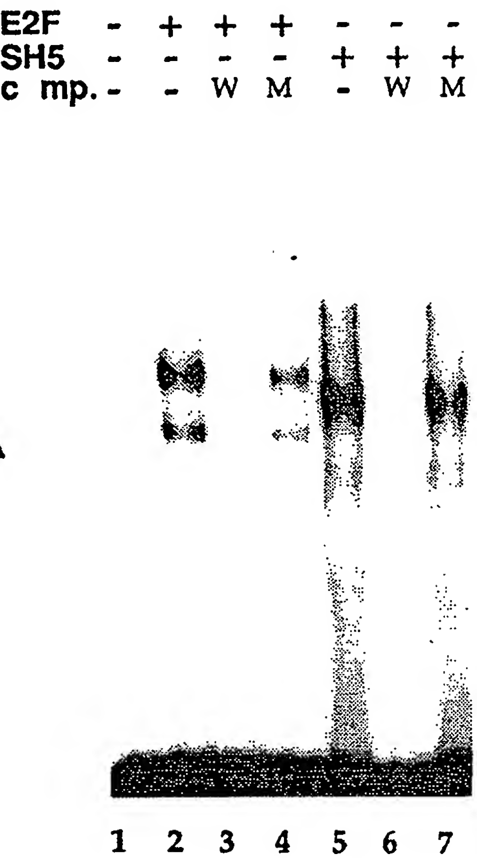


FIG. 7A

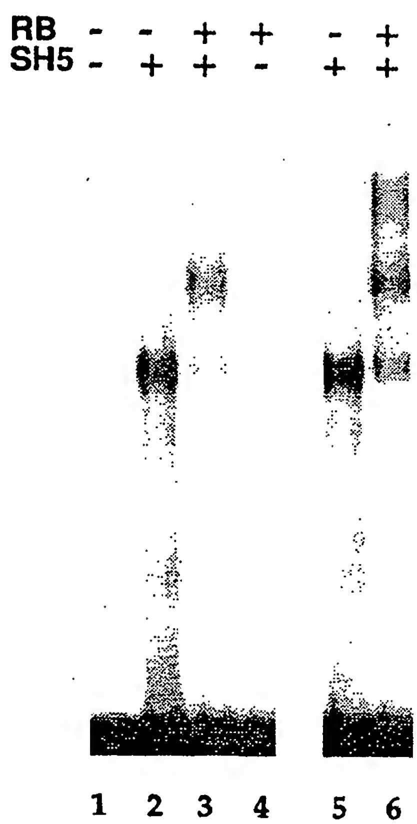


FIG. 7B

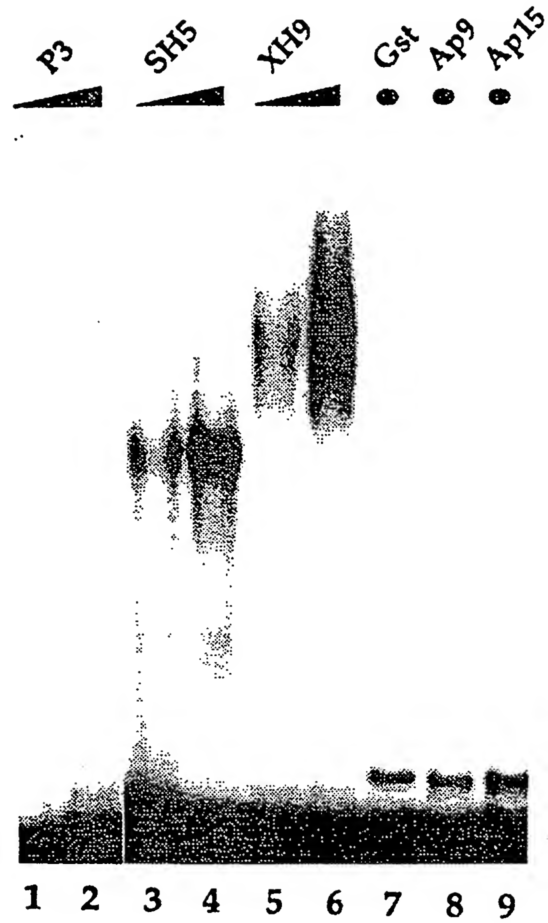

P3
SH5
XH9
Gst
Ap9
Ap15
1 2 3 4 5 6 7 8 9

FIG. 7C

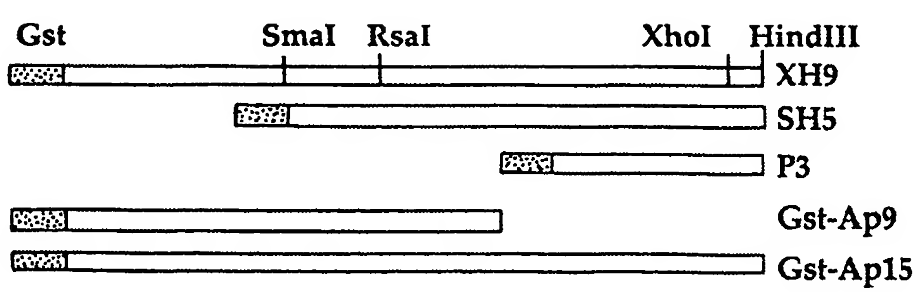

Gst
SmaI
RsaI
XhoI
HindIII
XH9
SH5
P3
Gst-Ap9
Gst-Ap15

FIG. 7D

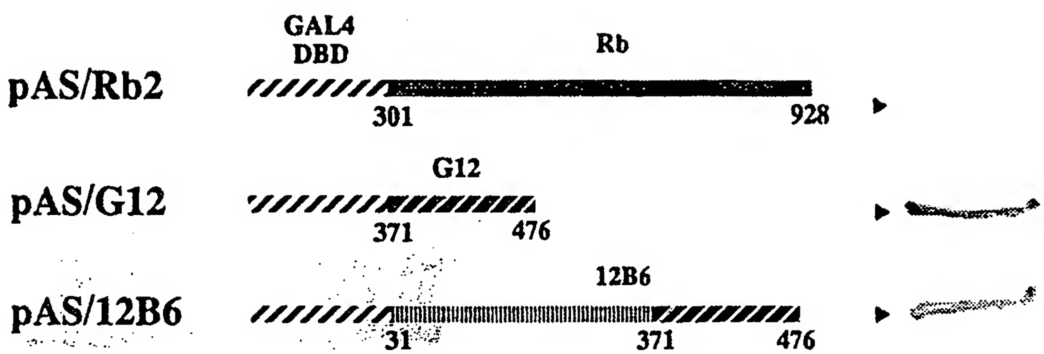

GAL4
DBD
Rb
pAS/Rb2
301
928
G12
pAS/G12
371
476
12B6
pAS/12B6
31
371
476

FIG. 8

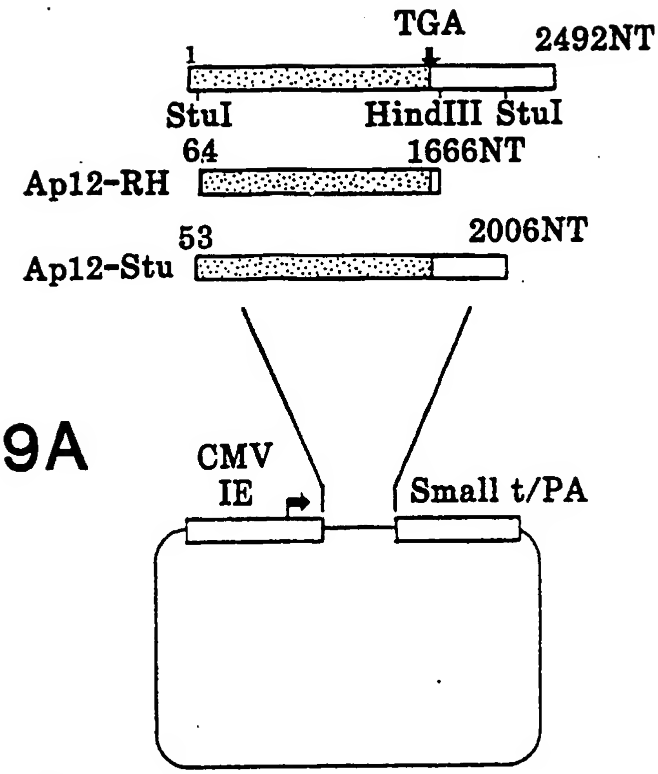

TGA
1
2492NT
StuI
HindIII StuI
64
1666NT
Ap12-RH
53
2006NT
Ap12-Stu
CMV
IE
Small t/PA

FIG. 9A

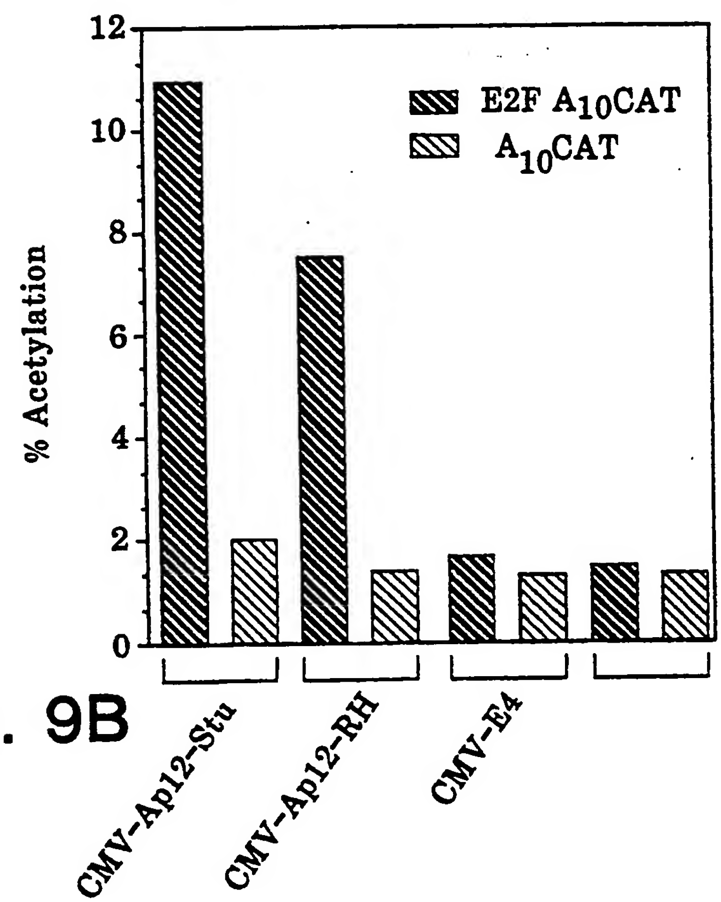

% Acetylation
12
10
8
6
4
2
0
E2F A_{10}CAT
A_{10}CAT
CMV-Ap12-Stu
CMV-Ap12-RH
CMV-E4

FIG. 9B

KbAp2p

10 20 30 40 50 60 70 80

CGCCTTGACCTTGCTGGGAATGCTCGGTCAGACAAGGGCAGCATGTCTGAAGACTGTGGGCCAGGAACCTCCGGGGAGCT 80
GGGCGGCTGAGGCGATCAAAATTGAGCCAGAGGATCTGGACATCATTCAGGTCACCGTCCCAGACCCCTCGCCAACCTCT 160
GAGGAAATGACAGACTCG 178

RbAp2r

10 20 30 40 50 60 70 80

TTTTTTACTTATTTAAAAAGGCCTTGGTGGCAGGAATATAGTGTAAAAATCATTGGAAAAACTAAAAGGCATCGATACAT 80
ATCCGAATATACATTTTGTACATAAATTACATTTCCTTTAGTCTTTCTGAGTGAGGTCCTGATTCAGTACT 151

FIG. 10

RbAp8p

10 20 30 40 50 60 70 80

TTTACGACAGAGCACTATTGCCAAGCGTTCAAATGCAGCACCATTAAGTAACACAAAAAAAGCATCTGGGAAGACTGTAT 80
CTACTGCTAAAGCAGGAGTGAAACAACCAGAAAGGAGTCAGGTTAAAGAAGAAGTATGTATGTCACTGAAACCTGAGTAC 160
CATAAGGAGAATAGAAGGTGCAGCCGAAATAGCGGACAAATTGAAGTGGATACCTGAAGTATCAGTGTCTTCAAGTCATT 240
CTTCAGTGTCATCTT 255

RbAp8r

10 20 30 40 50 60 70 80

GAATTCAACTGTAGCTTGGTTTTCCAAAGTATCTGGATCTAGTATTTCAGTCTTTTTGTCTTCTTCAGCACAACATTTTA 80
CACAGACATATTCTTTGTCTTCCTCGCCCATCTGCTGTGCTTGAGAAAGACTTAACCCAACACAATCACCATGAAACCAG 160
TCATCACATCTCCACAGCCAACCATAACTGTTGCATGTGTTTTTGCAAACCACACTGTTGCTGGAGTCACATATATTCGT 240
TCAAT 245

FIG. 11

RbAp15p

10 20 30 40 50 60 70 80

GAATTCAGTGGAGCACCAGTAGAAGGTGCAGGAGAAGAGGCATTGACTCCATCAGTTCCTATAAATAAAGGTCCCAAACC 80
TAAGAGGGAGAAGAAGGAGCCTGGTACCAGAGTGAGAAAAACACCTACATCATCTGGTAAACCTAGTGCAAAGAAAGTGA 160
AGAAACGGAATCCTTGGTCAGATGATGAATCCAAGTCAGAAAGTGATTTGGAAGAAACAGAACCTGTGGTTATTCCAAGA 240
GATTCTTTGCTTAGGAGAGCAGCAGCCGAAAGACCTAAATACACATTTAATTTCTCAGAAGAAGAGGATGATGATGCTGA 320
TGATGATGATGATGACAATAATGATTTAGAGGAATTGAAAGTTAAAGCATCTCCCATAACAAATGATGGGGAAGATGAAT 400

410 420 430 440 450 460 470 480

TTGTTCCTTCAGATGGGTTAGATAAAGATGAATATACATTTTCACCAGGCAAATCAAAAGCCTCACCAGAAAAATCTTTG 480
CATGACAAAAAAAGTCAGGATTTTGGAAATCTCTTCTCATTTCCTTCATATTCTCAGAAGTCAGAAGATGATTCAGCTAA 560
ATTTGACAGTAATGAAGAAGATTCTGCTTCTGTTTTTTCACCATCATTTGGTCTGAAACAGACAGATAAAGTTCCAAGTA 640
AAACGGTAGCTGCTAAAAAGGGAAAACCGTCTTCAGATACAGTCCCTA 688

RbAp15r

10 20 30 40 50 60 70 80

GCAATGTTTAATTAAGTGGGGAAAGAGCACAAACATTTTTCAACAAATACTTGTGTTGTCCTTTTGTCTTCTCTGTCTCA 80
GACCTTTTGTACATCTGGCTTATTTTAATGTGATGATGTAATTGACCGTTTTTTATTATTGTGGTAGGCCTTTTAACATT 160
TTGTTCTTACACATACAGTTTTATGCTCTTTTTTACTCATTGAAATGTCACGTACTGTCTGATTGGCTTGTAGAATTGGT 240
TATAGACTGCCGTGCATTAGCACAGATTTTAATTGTCATGGTTACAAACTACAGACCTGCTTTTTGAAATGAAATTTAAA 320
CATTAAAAATGGAACTGTGAAAAAAAAA 348

FIG. 12

RbAp4

10 20 30 40 50 60 70 80

GAATTCCGGGCCAAGAAGCCTAATGAGAAAAACAAACCACTTGATAATAAGGGAGAAAAAAGAAAAAGAAAAACTGAAGA 80
AAAAGGCGTAGATAAAGATTTTGAGTCTTCTTCAATGAAAATCTCGAAACTAGAAGTGACTGAAATAGTGAAACCATCAC 160
CAAAGCGCAAAATGGAACCTGATACTGAAAAAATGGATAGGACCCCTGAAAAGGACAAAATTTCTTTAAGTGCGCCAGCC 240
AAAAAAATCAAACTCAACAGAGAAACTGGGAAGAAAATTGGAAGTACAGAAAATATATCAAACACAAAAGAACCCTCTGA 320
AAAATTGGAGTCAACATCTAGCAAAGTTAAACAAGAAAAAGTCAAAGGAAAGGTCAGACGAAAAGTGACTGGAACTGAAG 400

410 420 430 440 450 460 470 480

GATCCAGCTCAACTCTGGTGGATTACaCCaGTACGAGCTCAACTGGAGGCAGTCCTGTGCGGAAATCTGAAGAAAAAACA 480
GATACAAAGCGAACTGTGATTAAAACGATGGAAGAATATAATAATGACAATACCGCGCCACGTGAAGATGTTATCATTAT 560
GATTCAGGTTCCTCAATCCAAATGGGATAAAGATGACTTTGAATCTGAAGAAGAAGATGTTAAATCCACACAGCCTATAT 640
CAAGTGTAGGAAAACCTGCTAGTGTTATAAAAAATGTTAGTACAAAGCCATCAAATATAGTCAAGTATCCTGAGAAAGAA 720
AGTGAGCCATCCGAGAAAATTCAGAAATTCACCAAGGACGTGAGCCATGAAATCATACAACATGAGGTTAAAAGTTCAAA 800

810 820 830 840 850 860 870 880

AAACTCTGCATCTAGTGAAAAAGGGAAAACCAAAGATCGAGATTATTCAGTGTTGGAAAAGGAGAACCCTGAAAAGAGGA 880
AGAACAGCACTCAGCCAGAGAAAGAGAGTAATTTGGACCGTCTGAATGAACAAGGAAATTTTAAAAGTCTGTCTCAATCT 960
TCCAAAGAGGCTAGAACGTCAGATAAACATGATTCCACTCGTGCTTCCTCAAATAAAGACTTCACTCCCAATAGAGACAA 1040
AAAAACTGACTATGACACCAGAGAGTATTCAAGTTCCAAAcgTAGAGATGAAAAGAATGAATTAACAAGACGAAAAGACT 1120
CTCCTTCTCGGAATAAAGATTCTGCATCTGGACAGAAAAATAAACCAAGGGAAGAGAGAGATTTGCCTAAAAAAGGAACA 1200

1210 1220 1230 1240 1250 1260 1270 1280

GGAGATTCCAAAAAAAGTAATTCTAGTCCCTCAAGAGACAGAAAACCTCATGATCACAAAGCCACTTATGATACTAAACG 1280
GCCAAATGAAGAGACAAAATCTGTAGATAAAAATCCTTGTAAGGATCGTGAGAAGCATGTATTAGAAGCAAGGAACAATA 1360
AAGAGTCAAGTGGCAATAAAcTaCTTTATATACTTAACCCACCAGAGAcAcAGGTTGAAAAAGAGCAAATTACTGGGCAA 1440
ATTGACAAGAGTACTGTCAAGCCTAAACCCCAGTTAAGTCATTCCTCTAGACTTTCCTCTGACTTAACTAGAGAAACTCA 1520
TGAAGCTGCTTTTGAACCAGACTATAATGAAAGTGACAGTGAAAGTAATGTTTCTGTAAAAGAAGAGGAATCTTCAGGAA 1600

1610 1620 1630 1640 1650 1660 1670 1680

ACATTTCTAAGGACCTGAAAGATAAAATAGTGGAGAAAGCAAAAGAGAGCCTGGACACAGCAGCAGTTGTCCAGGTGGGC 1680
ATAAGCAGGAATCAGAGCCACAGCAGCCCCAGCGTCAGCCCCAGCAGAAGCCACAGTCCTTCTGGAAGCCAGACCCGAAG 1760
CCACAGTAGCAGTGCCAGCTCAGCAGAAAGTCAGGACAGC 1800

FIG. 13

RbAp10

```
        10        20        30        40        50        60        70        80
GAATTCCGGCCGGAATTAATTCCGGGGATTTCCTGGGGAATCAGGAAGATATCCATAATCTTCAACTGCGGGTAAAAGAG 80
ACATCAAATGAGAATTTGAGATTACTTCATGTGATAGAGGACCGTGACAGAAAAGTTGAAAGTTTGCTAAATGAAATGAA 160
AGAATTAGACTCAAAACTCCATTTACAGGAGGTACAACTAATGACCAAAATTGAAGCATGCATAGAATTGGAAAAAATAG 240
TTGGGGAACTTAAGAAAGAAAACTCAGATTTAAGTGAAAAATTGGAATATTTTTCTTGTGATCACCAGGAGTTACTCCAG 320
AGAGTAGAAACTTCTGAAGGCCTCAATTCTGATTTAGAAATGCATGCAGATAAATCATCACGTGAAGATATTGGAGATAA 400
       410       420       430       440       450       460       470       480
TGTGGCCAAGGTGAATGACAGCTGGAAGGAGAGATTTCTTGATGTGGAAAATGAGCTGAGTAGGATCAGATCGGAGAAAG 480
CTAGCATTGAGCATGAAGCCCTCTACCTGGAGGCTGACTTAGAGGTAGTTCAAACAGAGAAGCTATGTTTAGAAAAAGAC 560
AATGAAAATAAGCAGAAGGTTATTGTCTGCCTTGAAGAAGAACTCTCAGTGGTCACAAGTGAGAGAAACCAGCTTCGTGG 640
AGAATTAGATACTATGTCAAAAAAAACCACGGCACTGGATCAGTTGTCTGAAAAAATGAAGGAGAAAACACAAGAGCTTG 720
AGTCTCATCAAAGTGAGTGTCTCCATTGCATTCAGGTGGCAGAGGCAGAGGTGAAGGAAAAGACGGAACTCCTTCAGACT 800
       810       820       830       840       850       860       870       880
TTGTCCTCTGATGTGAGTGAGCTGTTAAAAGACAAAACTCATCTCCAGGAAAAGCTGCAGAGTTTGGAAAAGGACTCACA 880
GGCACTGTCTTTGACAAAATGTGAGCTGGAAAACCAAATTGCACAACTGAATAAAGAGAAAGAATTGCTTGTCAAGGAAT 960
CTGAAAGCCTGCAGGCCAGACTGAGTGAATCAGATTATGAAAAGCTGAATGTCTCCAAGGCCTTGGAGGCCGCACTGGTG 1040
GAGAAAGGTGAGTTCGCATTGAGGCTGAGCTCAACACAGGAGGAAGTGCATCAGCTGAGAAGAGGCATCGAGAAACTGAG 1120
AGTTCGCATTGAGGCCGATGAAAAGAAGCAGCTGCACATCGCAGAGAAACTGAAAGAACGCGAGCGGGAGAATGATTCAC 1200
      1210      1220      1230      1240      1250      1260      1270      1280
TTAAGGATAAAGTTGAGAACCTTGAAAGGGAATTGCAGATGTCAGAAGAAAACCAGGAGCTAGTGATTCTTGATGCCGAG 1280
AATTCCAAAGCAGAAGTAGAGACTCTAAAAACACAAATAGAAGAGATGGCCAGAAGCCTGAAAGTTTTTGAATTAGACCT 1360
TGTCACGTTAAGGTCTGAAAAAGAAAATCTGACAAAACAAATACAAGAAAAACAAGGTCAGTTGTCAGAACTAGACAAGT 1440
TACTCTCTTCATTTAAAAGTCTGTTAGAAGAAAAGGAGCAAGCAGAGATACAGATCAAAGAAGAATCTAAAACTGCAGTG 1520
GAGATGCTTCAGAATCAGTTAAAGGAGCTAAATGAGGCAGTAGCAGCCTTGTGTGGTGACCAAGAAATTATGAAGGCCAC 1600
      1610      1620      1630      1640      1650      1660      1670      1680
AGAACAGAGTCTAGACCCACCAATAGAGGAAGAGCATCAGCTGAGAAATAGCATTGAAAAGCTGAGAGCCCGCCTAGAAG 1680
CTGATGAAAAGAAGCAGCTCTGTGTCTTACAACAACTGAAGGAAAGTGAGCATCATGCAGATTTACTTAAGGGTAGAGTG 1760
GAGAACCTTGAAAGAGAGCTAGAGATAGCCAGGACAAACCAAGAGCATGCAGCTCTTGAGGCAGAGAATTCCAAAGGAGA 1840
GGTAGAGACCCTAAAAGCAAAAATAGAAGGGATGACCCAAAGTCTGAGAGGTCTGGAATTAGATGTTGTTACTATAAGGT 1920
CAGAAAAAGAAAATCTGACAAATGAATTACAAAAAGAGCAAGAGCGAATATCTGAATTAGAAATAATAAATTCATCATTT 2000
      2010      2020      2030      2040      2050      2060      2070      2080
GAAAATATTTTGCAAGAAAAAGAGCAAGAGAAAGTACAGATGAAAGAAAAATCAAGCACTGCCATGGAGATGCTTCAAAC 2080
ACAATTAAAAGAGCTCAATGAGAGAGTGGCAGCCCTGCATAATGACCAAGAAGCCTGTAAGGCCAAAGAGCAGAATCTTA 2160
GTAGTCAAGTAGAGTGTCTTGAACTTGAGAAGGCTCAGTTGCTACAAGGCCTTGATGAGGCCAAAAATAATTATATTGTT 2240
TTGCAATCTTCAGTGAATGGCCTCATTCAAGAAGTAGAAGATGGCAAGCAGAAACTGGAGAAGAAGGATGAAGAAATCAG 2320
TAGACTGAAAAATCAAATTCAAGACCAAGAGCAGCTTGTCTCTAAACTGTCCCAGGTGGAAGGAGAGCACCAACTTTGGA 2400
      2410      2420      2430      2440      2450      2460      2470      2480
AGGAGCAAAACTTAGAACTGAGAAATCTGACAGTGGAATTGGAGCAGAAGATCCAAGTGCTACAATCCAAAAATGCCTCT 2480
TTGCAGGACACATTAGAAGTGCTGCAGAGTTCTTACAAGAATCTAGAGAATGAGCTTGAATTGACAAAAATGGACAAAAT 2560
GTCCTTTGTTGAAAAAGTAAACAAAATGACTGCAAAGGAAACTGAGCTGCAGAGGGAAATGCATGAGATGGCACAGAAAA 2640
CAGCAGAGCTGCAAGAAGAACTCAGTGGAGAGAAAAATAGGCTAGCTGGAGAGTTGCAGTTACTGTTGGAAGAAATAAAG 2720
AGCAGCAAAGATCAATTGAAGGAGCTCACACTAGAAAATAGTGAATTGAAGAAGAGCCTAGATTGCATGCACAAAGACCA 2800
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FIG. 14A

RbAp10 CONT.

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     2810      2820      2830      2840      2850      2860      2870      2880
GGTGGAAAAGGAAGGGAAAGTGAGAGAGGAAATAGCTGAATATCAGCTACGGCTTCATGAAGCTGAAAAGAAACACCAGG 2880
CTTTGCTTTTGGACACAAACAAACAGTATGAAGTAGAAATCCAGACATACCGAGAGAAATTGACTTCTAAAGAAGAATGT 2960
CTCAGTTCACAGAAGCTGGAGATAGACCTTTTAAAGTCTAGTAAAGAAGAGCTCAATAATTCATTGAAAGCTACTACTCA 3040
GATTTTGGAAGAATTGAAGAAAACCAAGATGGACAATCTAAAATATGTAAATCAGTTGAAGAAGGAAAATGAACGTGCCC 3120
AGGGGAAAATGAAGTTGTTGATCAAATCCTGTAAACAGCTGGAAGAGGAAAAGGAGATACTGCAGAAAGAACTCTCTCAA 3200
     3210      3220      3230      3240      3250      3260      3270      3280
CTTCAAGCTGCACAGGAGAAGCAGAAAACAGGTACTGTTATGGATACCAAGGTCGATGAATTAACAACTGAGATCAAAGA 3280
ACTGAAAGAAACTCTTGAAGAAAAAACCAAGGAGGCAGATGAATACTTGGATAAGTACTGTTCCTTGCTTATAAGCCATG 3360
AAAAGTTAGAGAAAGCTAAAGAGATGTTAGAGACACAAGTGGCCCATCTGTGTTCACAGCAATCTAAACAAGATTCCCGA 3440
GGGTCTCCTTTGCTAGGTCCAGTTGTTCCAGGACCATCTCCAATCCCTTCTGTTACTGAAAAGAGGTTATCATCTGGCCA 3520
AAATAAAGCTTCAGGCAAGAGGCAAAGATCCAGTGGAATATGGGAGAATGGTGGAGGACCAACACCTGCTACCCCAGAGA 3600
     3610      3620      3630      3640      3650      3660      3670      3680
GCTTTTCTAAAAAAAGCAAGAAAGCAGTCATGAGTGGTATTCACCCTGCAGAAGACACGGAAGGTACTGAGTTTGAGCCA 3680
GAGGGACTTCCAGAAGTTGTAAAGAAAGGGTTTGCTGACATCCCGACAGGAAAGACTAGCCCATATATCCTGCGAAGAAC 3760
AACCATGGCAACTCGGACCAGCCCCCGCCTGGCTGCACAGAAGTTAGCGCTATCCCCACTGAGTCTCGGCAAAGAAAATC 3840
TTGCAGAGTCCTCCAAACCAACAGCTGGTGGCAGCAGATCACAAAAGGTCAAAGTTGCTCAGCGGAGCCCAGTAGATTCA 3920
GGCACCATCCTCCGAGAACCCACCACGAAATCCGTCCCAGTCAATAATCTTGCTGAGAGAAGTCCGACTGACAGCCCCAG 4000
     4010      4020      4030      4040      4050      4060      4070      4080
AGAGGGCCTGAGGGTCAAGCGCCGGCGACTTGTCCCCAGCCCCAAAGCTGGACTGGAGTCCAAGGGCAGTGAGAACTGTA 4080
AGGTCCAGTGAAGGCACTTTGTGTGTCAGTACCCCTGGGAGGTGCCAGTCATTGAATAGATAAGGCTGTGCCTACAGGAC 4160
TTCTCTTTAGTCAGGGCATGCTTTATTAGTGAGGAGAAAACAATTCCTTAGAAGTCTTAAATATATTGTACTCTTTAGAT 4240
CTCCCATGTGTAGGTATTGAAAAAGTTTGGAAGCACTGATCACCTGTTAGCATTGCCATTCCTCTACTGCAATGTAAATA 4320
GTATAAAGCTATGTATATAAAGCTTTTTGGTAATATGTTACAATTAAAATGACAAGCACTATATCACAATCTCTGTTTGT 4400
     4410      4420      4430      4440      4450      4460      4470      4480
ATGTGGGTTTTACACTAAAAAAATGCAAAACACATTTTATTCTTCTAATTAACAGCTCCTAGGAAAATGTAGACTTTTGC 4480
TTTATGATATTCTATCTGTAGTATGAGGCATGGAATAGTTTTGTATCGGGAATTTCTCAGAGCTGAGTAAAATGAAGGAA 4560
AAGCATGTTATGTGTTTTTAAGGAAAATGTGCACACATATACATGTAGGAGTGTTTATCTTTCTCTTACAATCTGTTTTA 4640
GACATCTTTGCTTATGAAACCTGTACATATGTGTGTGTGGGTATGTGTTTATTTCCAGTGAGGGCTGCAGGCTTCCTAGA 4720
GGTGTGCTATACCATGCGTCTGTCGTTGTGCTTTTTTCTGTTTTTAGACCAATTTTTTACAGTTCTTTGGTAAGCATTGT 4800
     4810      4820      4830      4840      4850      4860      4870      4880
CGTATCTGGTGATGGATTAACATATAGCCTTTGTTTTCTAATAAAATAGTCGCCTTCGTAAAAAAAAA 4868
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FIG. 14B

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 172.3, 240.2, 320.1; 436/501, 536; 530/350, 388.15, 389.1; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
Search terms: retinoblastoma-associated polypeptide, retinoblastoma binding, E2F factor, transcription factor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Cell, Volume 64, issued 08 February 1991, W.G. Kaelin, Jr. et al, "Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product", pages 521-532, see entire document.	6-8, 21
X	Nature, Volume 352, issued 18 July 1991, D. Defeo-Jones et al, "Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product", pages 251-254, see entire document.	1-13, 20-21
X	Cell, Volume 70, issued 24 July 1992, W.G. Kaelin, Jr. et al, "Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties", pages 351-364, see entire document.	1-21
X	Cell, Volume 70, issued 24 July 1992, K. Helin et al, "A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F", pages 337-350, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date f the actual completion of the international search	Date of mailing of the international search report
18 FEBRUARY 1994	07 MAR 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DIAN C. JACOBSON Jill Warden for
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Molecular and Cellular Biology, Volume 12, Number 10, issued October 1992, S.K. Ray et al, "Identification of a 60-kilodalton Rb-binding protein, RBP60, that allows the Rb-E2F complex to bind DNA", pages 4327-4333, see entire document.	6-8, 21
Y	Science, Volume 258, issued 16 October 1992, J.R. Nevins, "E2F: A link between the Rb tumor suppressor protein and viral oncoproteins", pages 424-429, see pages 427-428.	1-13, 20-21
Y	Nature, Volume 352, issued 18 July 1991, L.R. Bandara et al, "Cyclin A and the retinoblastoma gene product complex with a common transcription factor", pages 249-251, see entire document.	6-8, 21
P,X	Biochemical and Biophysical Research Communications, Volume 194, Number 2, issued 30 July 1993, A.A. Ali et al, "Retinoblastoma gene product-associated proteins in human colon cancer cell lines", pages 848-854, see entire document.	1-21

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

CO7H 21/04; C07K 13/00, 15/28; C12N 5/00, 5/10, 15/00, 15/12, 15/85, 15/86; G01N 33/53

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 69.1, 172.3, 240.2, 320.1; 436/501; 530/350, 388.15, 389.1; 536/23.5